GENES UNDERLYING SOCIAL INFLUENCE ON D. MELANOGASTER CLOCKS

By: Jayed (Jade) Atallah

Doctor of Philosophy - Cell and Systems Biology
Department of Cell and Systems Biology - University of Toronto
Department of Biology - University of Toronto Mississauga

2015

ABSTRACT

Social influence on an individual is found in many species. The mediation of such effects is based on communication, involving the generation, receipt, and integration of signals that modulate an organism’s behaviour. The fruit fly Drosophila melanogaster has played a significant role in understanding the mechanisms underlying social influences. One emerging theme is that an individual’s circadian system is highly responsive to the social surroundings and could mediate such influence. Yet a systematic search for genes underlying social cues that affect an individual’s internal state, has been hampered by the lack of a reliable and robust ‘social screen’. In this study, the known aspects of social influence were assimilated to create an assay which monitors circadian gene expression in a focal fly when interacting with a partner (stimulus fly). The stimulus fly was either wild-type, or harbored an unknown (forward genetics) or known (reverse genetics) mutation. Deviations in the circadian signal of the focal fly were used to identify novel genes in the stimulus fly that underlie social interactions. Through the forward genetic approach, 2552 chemically mutagenized lines were established, of which 10 novel mutations affected social interactions. Through the reverse genetic approach, several candidate genes altered social interactions, one of which is homologous to a mammalian limbic associated membrane gene. This limbic-homologue and an EMS mutant were further characterized to identify potential pathways through which social effects are manifested. Along
with altered locomotor activity, both displayed significantly altered hydrocarbon profiles. This suggests that social influence likely operates through multiple pathways, including olfaction and the clock system. Despite the assay being conducted in a light-dark environment, phase shifts, changes in expression levels, and/or changes in the rhythmicity index were all detected. This suggests that social effect on the circadian system is a potent modulator, even when operating within the confines of a much stronger Zeitgebers such as light. The implications of the assay and collected data were discussed. A model framework through which the mechanisms of social interactions can be further studied was also synthesized.
ACKNOWLEDGMENTS

A thank you is in order for several individuals:

Dr. Joel Levine, an exceptional mentor, inspiring person, and great friend. To him I owe most of my development as a scientist and becoming a better person, and without whom, I would question my accomplishments.

My committee members, Dr. Marla Sokolowski and Dr. Bryan Stewart, who have been generous in offering their time, expert advice, and encouragement over the course of my research. The same applies for my external examiners, Dr. Helen Rodd and Dr. Charalambos P. Kyriacou. Thank you!

Dr. Jean-Christophe Billeter who was my mentor at the bench and to whom I owe a significant portion of my development as a scientist.

Dr. Joshua Krupp who was always available for critical advice and whose work ethics I’ll always look up to.

Dr. Jonathan Schneider who has and continues to be an ideal colleague, and exceptional scientist and person. His feedback, advice, and continuous willingness to share his skills have been absolutely critical throughout my years at the Levine Laboratory. Thank you so much Jon.

Previous and current Levine Laboratory members, some of whom have been great friends, and whose encouragement was much valued. These wonderful individuals include Adrienne Chu, Dr. Richard Dunbar-Yaffe, Dr. Hania Pavlou, and Samyukta Jagadeesh.

Ian Buglass and Carolyn Moon who perform their jobs so refreshingly well. Thank you for being fantastic staff members.

Other members of the Biology Department at the University of Toronto Mississauga: Dr. Sasa Stefanovic and Dr. Bryan Stewart, inspiring professors and whose vision and work as chairs of Biology has made our department prideful to belong to; Dr. Helen Wagner, Dr. Linda Kohn, and Dr. Jim Anderson for always being genuinely supportive faculty members, as well as for their invested efforts in the University of Toronto Mississauga Biology Postgraduate Mentoring Program; Dr. Tim Westwood for his prompt readiness to provide scientific advice whenever necessary; Dr. Espie for always supporting my teaching aspirations while making sure my research is not compromised.
Few great individuals whose encouragement was always motivating in stressful times: Paul Georgiou, Suleiman Warwar, Harrod ling, Gizele Toutounji, Janice Ting, as well as Pat and Joe Catalano.

My family: Joseph Saliba, Joanna Atallah, Matile Kaadi, Roger Patrick, Tam Khuu, Jessika Atallah, and Adel Atallah. Thank you for your heart warming care and continuous support and encouragement.

The one person who has taught and helped me to always aim highest, Adona El-Khoury. Thank you Mother.

The authors of ‘An introduction to genetic analysis’ (Griffiths et al., 1993), whose elaboration on the research underlying fundamental concepts in genetics, sparked my first interest in biological research.

Thank you all.
# Table of Contents

Abstract .............................................................................................................. ii
Acknowledgments .............................................................................................. iv
Table of Contents .............................................................................................. vi
List of Figures .................................................................................................... ix
List of Tables .................................................................................................... xi
List of Appendices ............................................................................................ xii
List of Acronyms ............................................................................................... xiii
Forward ................................................................................................................ 1

Chapter 1 One, Two, and Many—A Perspective on What Groups of *Drosophila melanogaster* Can Tell Us About Social Dynamics .......................................................... 3
1.1 Abstract ....................................................................................................... 3
1.2 Introduction .................................................................................................. 3
1.3 Caveat .......................................................................................................... 6
1.4 The Behavioral Effects Of Social Context ......................................................... 6
   1.4.a Social Learning And Memory ................................................................. 6
   1.4.b Social Synchronizing Of Activity And Rest .............................................. 8
   1.4.c Aggression ............................................................................................. 10
   1.4.d Mating, Paternity, And Offspring ............................................................ 12
1.5 Conclusion .................................................................................................... 15

Chapter 2 Mechanisms Of Social Influence On *D. Melanogaster* Locomotor Activity .................................................. 18
2.1 *D. melanogaster* Clocks .............................................................................. 19
2.2 *D. melanogaster* Locomotor Activity ........................................................ 20
2.3 Immediate Responses to the Social Environment ........................................ 20
   2.3.a Pairwise gender-specific activity and close-proximity rhythms in DD ............. 20
   2.3.b Pairwise gender-specific activity rhythms in LD ....................................... 23
2.4 Long-term Responses to the Social Environment ......................................... 27
   2.4.a Pairwise MM influence on long-term activity rhythms .............................. 27
   2.4.b Influence of heterosexual groupings on long-term locomotor activity .............. 29
   2.4.c Influence of isolation and group composition on long-term locomotor activity ............................................................. 31
2.5 Conclusions Regarding Social effects on *D. melanogaster* Locomotor Activity .................................................. 33
5.6 Chapter 5 Tables ........................................................................................................... 84
Chapter 6 Reverse Genetic approach .................................................................................. 87
  6.1 Screening D. melanogaster Homologues Of Mammalian LSAMP ................................. 87
  6.2 Secondary Characterization of CG42343 Candidate ...................................................... 88
    6.2.a Locomotor Activity .................................................................................................. 88
    6.2.b Clock Genes Expression ......................................................................................... 89
    6.2.c Clock-related Circuitry ............................................................................................. 89
    6.2.d Hydrocarbon Profiles and Expression of the Hydrocarbon Synthesis Enzyme DESAT .............................................................................................................. 89
    6.2.e Courtship and Mating Behaviour .......................................................................... 90
  6.3 Chapter 6 Figures ......................................................................................................... 92
  6.4 Chapter 6 Tables ......................................................................................................... 98
Chapter 7 Discussion ......................................................................................................... 99
  7.1 Social Clock Plasticity Likely Functions Within The Confines Of Photoperiod Input .......... 99
  7.2 Multiple Loci, Likely Operating In Different Pathways Including the Clock Mechanism, Might be Involved In Inflicting Social Influence On Conspecifics ................................................................. 100
  7.3 Secondary Characterization of Line 192-X .................................................................. 103
  7.4 Secondary Characterization of Limbic Candidate ......................................................... 105
  7.5 Potential Mechanisms Through Which Line 192-X and the Limbic Candidate Influence A Companion’s Clock ....................................................................................................................... 106
  7.6 Chapter 7 Figures ....................................................................................................... 108
Chapter 8 Conclusion ......................................................................................................... 109
Chapter 9 Methods ............................................................................................................ 111
  9.1 Fly Rearing ................................................................................................................... 111
  9.2 TC Bioluminescence Monitoring .................................................................................. 111
  9.3 Locomotor Activity Testing ......................................................................................... 111
  9.4 Cuticular Hydrocarbon Analysis ................................................................................ 112
  9.5 Courtship Behaviour Analysis ..................................................................................... 112
References ........................................................................................................................... 113
LIST OF FIGURES

FIGURE 3.1 | OENOCYTES PRODUCE CUTICULAR HYDROCARBONS ............................................................. 45
FIGURE 3.2 | HYDROCARBONS REGULATE SEXUAL ATTRACTIVENESS ......................................................... 46
FIGURE 3.3 | 7,11-HD REGULATES COURTSHIP BETWEEN DROSOPHILA SPECIES .................................. 47
FIGURE 3.4 | MATING BETWEEN D. MELANOGASTER FEMALES AND D. SIMULANS MALES IS PREVENTED BY 7,11-HD .... 47
FIGURE 4.1 | SCREEN PARADIGM DESCRIPTION ......................................................................................... 57
FIGURE 4.2 | EXAMPLE FIGURE ILLUSTRATING THE CUSTOMIZED AND AUTOMATED ANALYSIS OF PER-DRIVEN BIOLUMINESCENCE USING FLIES AS REPLICATES ......................................................... 58
FIGURE 4.3 | EXAMPLE FIGURE ILLUSTRATING THE CUSTOMIZED AND AUTOMATED ANALYSIS OF PER-DRIVEN BIOLUMINESCENCE USING INDIVIDUAL EXPERIMENTS AS REPLICATES .............................................................. 59
FIGURE 4.4 | ANALYSIS OF PER-DRIVEN BIOLUMINESCENCE OF SOLITARY REPORTER COMPARED TO A REPORTER CO-HOUS ED WITH A WILD-TYPE CONSPECIFIC USING FLIES AS REPLICATES .................................................. 60
FIGURE 4.5 | ANALYSIS OF PER-DRIVEN BIOLUMINESCENCE OF SOLITARY REPORTER COMPARED TO A REPORTER CO-HOUS ED WITH A WILD-TYPE CONSPECIFIC USING INDIVIDUAL EXPERIMENTS AS REPLICATES ........................................ 61
FIGURE 5.1 | DETERMINATION OF EMS MUTAGENIC EFFICIENCY BY SCORING FOR X-LETHAL MUTATIONS ................................................................. 72
FIGURE 5.2 | FLOWCHART INDICATING THE STAGES AT WHICH SCREENING, RE-TESTING, AND DIFFERENT GENETIC CROSSES WERE CONDUCTED .............................................................................................................. 73
FIGURE 5.3 | GENETIC CROSSING SCHEME EMPLOYED TO GENERATE MUTANT LINES ........................................ 74
FIGURE 5.4 | GENETIC CROSSING SCHEME EMPLOYED TO ESTABLISH LINES WITH MUTAGENIZED X CHROMOSOMES ONLY ................................................. 74
FIGURE 5.5 | GENETIC CROSSING SCHEME EMPLOYED TO ESTABLISH LINES WITH EITHER 2ND OR 3RD MUTANT CHROMOSOMES ............................................................. 74
FIGURE 5.6 | GENETIC CROSSING SCHEMES EMPLOYED TO ESTABLISH 20 INDIVIDUAL LINES HOMOZYGOUS FOR THE AUTOSOMES FROM EACH PUTATIVE 2ND AND 3RD CHROMOSOME MUTANT LINES ......................................................................................... 75
FIGURE 5.7 | COMPARISON OF SOME PARENT MUTANT LINES WITH MUTATIONS ON MORE THAN 1 CHROMOSOME TO DESCENDANT LINES WITH ONLY ONE MUTAGENIZED CHROMOSOME ........................................................................ 76
FIGURE 5.8 | EXAMPLE FIGURE ILLUSTRATING THE CUSTOMIZED AND AUTOMATED ANALYSIS OF LOCOMOTOR ACTIVITY ......................................................... 77
FIGURE 5.9 | ANALYSIS OF LOCOMOTOR ACTIVITY OF LINE 192-X COMPARED TO CONTROL IN LD ..................... 78
FIGURE 5.10 | ANALYSIS OF LOCOMOTOR ACTIVITY OF LINE 1414-X COMPARED TO CONTROL IN LD .................. 79
FIGURE 5.11 | ANALYSIS OF LOCOMOTOR ACTIVITY OF LINE 192-X COMPARED TO CONTROL IN DD ................ 80
FIGURE 5.12 | ANALYSIS OF LOCOMOTOR ACTIVITY OF LINE 1414-X COMPARED TO CONTROL IN DD ................ 81
FIGURE 5.13 | GENETIC CROSSING SCHEME EMPLOYED TO GENERATE RECOMBINANT LINES FOR EMS LINE 192-X OR AS A REFERENCE STRAIN ............................................................................................................................ 82
FIGURE 5.14 | GENETIC CROSSING SCHEME EMPLOYED TO INSERT BG-LUC, TIM-LUC, OR DESAT-LUC INTO THE BACKGROUND OF CANDIDATE LINES WITH MUTATION OF INTEREST MAPPING TO THE X CHROMOSOME ............................................................................. 82
FIGURE 5.15 | SPATIAL PROJECTION OF PDF-POSITIVE NEURONS IN LINE 192-X ............................................. 83
FIGURE 5.16 | ANALYSIS OF COURTSHIP BEHAVIOUR OF LINE 192-X .............................................................. 83
FIGURE 6.1 | IDENTIFICATION OF D. MELANOGASTER HOMOLOGUES OF MAMMALIAN LSAMP USING ENSEMBL ................................................................. 92
FIGURE 6.2 | ANALYSIS OF PER-DRIVEN BIOLUMINESCENCE OF REPORTER IN THE PRESENCE OF LIMBIC MUTANT LINE COMPARED TO A REPORTER CO-HOUS ED WITH A WILD-TYPE CONSPECIFIC ........................................................ 93
FIGURE 6.3 | ANALYSIS OF LOCOMOTOR ACTIVITY OF LIMBIC MUTANT LINE COMPARED TO CONTROL IN LD ........................................................ 94
FIGURE 6.4 | ANALYSIS OF LOCOMOTOR ACTIVITY OF LIMBIC MUTANT LINE COMPARED TO CONTROL IN DD 95
LIST OF TABLES

TABLE 2-1 | SUMMARY OF STUDIES INVESTIGATING THE INFLUENCE OF THE SOCIAL ENVIRONMENT ON LOCOMOTOR ACTIVITY. .....37
TABLE 2-2 | SUMMARY OF TREATMENTS INVESTIGATING THE ROLE OF DIFFERENT CLOCK NEURONS IN MEDIATING THE SOCIAL INFLUENCE OF MALE-FEMALE COHABITATION. ...............................................................................................................................37
TABLE 5-1 | SUMMARY OF LINES WITH CHROMOSOME-MAPPED MUTATIONS AND THEIR CORRESPONDING EFFECT ON THE REPORTER’S PER EXPRESSION. ........................................................................................................................................84
TABLE 5-2 | SUMMARY OF LINES WITH CHROMOSOME-MAPPED MUTATIONS AND THEIR CORRESPONDING LOCOMOTOR ACTIVITY PHENOTYPES........................................................................................................................................84
TABLE 5-3 | LOCOMOTOR ACTIVITY PHENOTYPES OF RECOMBINANTS LINES GENERATED FROM EMS CANDIDATE LINE 192-X. .....85
TABLE 5-4 | HYDROCARBON PROFILE OF LINE 192-X........................................................................................................................................86
TABLE 6-1 | HYDROCARBON PROFILE OF LIMBIC LINE WITH MANIPULATED CG42343 LOCUS (18681).........................................................98
LIST OF APPENDICES

Appendix I Phenotypes Of Candidate Lines in Social Screen .......................................................... 122
  A.I.1 Forward Genetic Approach Candidates .............................................................................. 122
  A.I.2 Reverse Genetic Approach Candidates ............................................................................. 143
    A I.2.a Limbic Candidates .................................................................................................. 143
    A I.2.b Other Candidates ................................................................................................. 153
Appendix II Locomotor Activity Phenotypes Of Select Candidate Lines .................................... 156
Appendix III MATLAB Codes ...................................................................................................... 162
Appendix IV Publications ............................................................................................................ 181
  A.IV.1 ................................................................................................................................. 181
  A.IV.2 ................................................................................................................................. 182
  A.IV.3 ................................................................................................................................. 183
Appendix V Permissions for Reproducing Published Manuscripts ........................................... 184
  A.V.1 Permission for reproducing (Schneider et al., 2012a).................................................... 184
  A.V.2 Permission for reproducing (Billeter et al., 2009)......................................................... 185
**LIST OF ACRONYMS**

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>7,11-HD</td>
<td>(7Z,11Z)-heptacosadiene</td>
</tr>
<tr>
<td>7,11-ND</td>
<td>(7Z,11Z)-nonacosadiene</td>
</tr>
<tr>
<td>7-T</td>
<td>(Z)-7-tricosene or C23:1(7)</td>
</tr>
<tr>
<td>Ace</td>
<td>acetylcholine esterase</td>
</tr>
<tr>
<td>ARM</td>
<td>anestheisa-resistant memory</td>
</tr>
<tr>
<td>Aru</td>
<td>Arouser</td>
</tr>
<tr>
<td>Clk</td>
<td>Clock</td>
</tr>
<tr>
<td>CRI</td>
<td>Courtship rest index</td>
</tr>
<tr>
<td>cry</td>
<td>cryptochrome</td>
</tr>
<tr>
<td>CS</td>
<td>Canton-S</td>
</tr>
<tr>
<td>CSisoX</td>
<td>Canton-S with isogenized x chromosome</td>
</tr>
<tr>
<td>CT</td>
<td>circadian time (constant dark conditions)</td>
</tr>
<tr>
<td>cVA</td>
<td>cis-vaccenyl acetate</td>
</tr>
<tr>
<td>cyc</td>
<td>Cycle</td>
</tr>
<tr>
<td>DD</td>
<td>Constant dark conditions</td>
</tr>
<tr>
<td>desat</td>
<td>Desaturase</td>
</tr>
<tr>
<td>DN1</td>
<td>Dorsal neurons</td>
</tr>
<tr>
<td>egr-1</td>
<td>early growth response</td>
</tr>
<tr>
<td>EMS</td>
<td>Ethane methyl sulfonate</td>
</tr>
<tr>
<td>F1</td>
<td>First Filial</td>
</tr>
<tr>
<td>F2</td>
<td>Second Filial</td>
</tr>
<tr>
<td>FF</td>
<td>Female-female</td>
</tr>
<tr>
<td>for</td>
<td>Foraging</td>
</tr>
<tr>
<td>fru</td>
<td>fruitless</td>
</tr>
<tr>
<td>GnRH</td>
<td>gonadotropin-releasing hormone</td>
</tr>
<tr>
<td>hid</td>
<td>head involution defective</td>
</tr>
<tr>
<td>IP(3)K</td>
<td>inositol 1,3,5 triphosphate kinase</td>
</tr>
<tr>
<td>LD</td>
<td>Light dark cycle</td>
</tr>
<tr>
<td>LL</td>
<td>Constant light conditions</td>
</tr>
<tr>
<td>ILN_v</td>
<td>Large Ventral lateral neurons</td>
</tr>
<tr>
<td>LN_d</td>
<td>Dorsal lateral neurons</td>
</tr>
<tr>
<td>LSAMP</td>
<td>limbic system-associated membrane protein</td>
</tr>
<tr>
<td>M-cells</td>
<td>Morning-cells</td>
</tr>
<tr>
<td>MF</td>
<td>Male-female</td>
</tr>
<tr>
<td>M-F+</td>
<td>Mutant males and WT females</td>
</tr>
<tr>
<td>MM</td>
<td>Male-male</td>
</tr>
<tr>
<td>MSDR</td>
<td>Male sex drive rhythm</td>
</tr>
<tr>
<td>Oe-</td>
<td>oenocyte-less</td>
</tr>
<tr>
<td>OR</td>
<td>Oregon-R</td>
</tr>
<tr>
<td>Term</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>Or83b</td>
<td>Or83b olfactory-receptor gene</td>
</tr>
<tr>
<td>ORN</td>
<td>Olfactory receptor neurons</td>
</tr>
<tr>
<td>PA</td>
<td>Presence absence</td>
</tr>
<tr>
<td>Pdf</td>
<td>Pigment-dispersing factor</td>
</tr>
<tr>
<td>Pdp1</td>
<td>Pdp1-epsilon</td>
</tr>
<tr>
<td>per</td>
<td>period</td>
</tr>
<tr>
<td>per0</td>
<td>Null period mutant</td>
</tr>
<tr>
<td>perL</td>
<td>Long period mutant</td>
</tr>
<tr>
<td>perS</td>
<td>Short period mutant</td>
</tr>
<tr>
<td>RI</td>
<td>Rhythmicity Index</td>
</tr>
<tr>
<td>RS</td>
<td>Rhythm Strength</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error of the Mean</td>
</tr>
<tr>
<td>SINs</td>
<td>Social Interaction Networks</td>
</tr>
<tr>
<td>sLNv</td>
<td>Small Ventral lateral neurons</td>
</tr>
<tr>
<td>SLR</td>
<td>Single locomotor activity rhythm</td>
</tr>
<tr>
<td>SNP</td>
<td>single nucleotide polymorphism</td>
</tr>
<tr>
<td>SSI</td>
<td>Socio-sexual interactions</td>
</tr>
<tr>
<td>TC</td>
<td>TopCount scintillation detector</td>
</tr>
<tr>
<td>tim</td>
<td>Timeless</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
</tr>
<tr>
<td>yw</td>
<td>yellow white</td>
</tr>
<tr>
<td>ZT</td>
<td>Zeitgeber</td>
</tr>
</tbody>
</table>
Animals perform numerous interactive behaviours to survive and reproduce, including but not limited to, foraging, feeding, nest building, mating, and parental care. These behaviours are modulated by genetic factors, environmental factors, and/or interactions between the two (Freeman and Herron, 2004; Sokolowski, 2001). Among environmental factors, the social environment plays a crucial role. The composition of the social environment can induce the release of behaviours such as courtship and mating, which do not always occur despite the continuous internal ability of an individual to manifest them (Tinbergen, 1990). For instance, although a male has a continuous internal ability to court, the release of this behaviour requires the presence of a female within his social environment. The social milieu not only regulates the manifestation of behaviours in an all or none fashion, but also modulates quantitative behavioural characteristics such as timing, duration, and intensity. This influence of the social surroundings on an individual’s behaviour is found in many species ranging from aggregating microbes to eusocial primates (Allee, 1938).

The potentially conserved mechanisms through which the social environment brings about behavioural modulation have been investigated in several species (reviewed in (Robinson et al., 2005; Schneider et al., 2012a)). For instance in the cichlid fish (*Astatotilapia burtoni*), removal of an alpha male alters a subordinate male’s reproductive behaviour. Such changes are linked to changes in gene expression (induction of *early growth response* (*egr-1*) expression), neuronal modulation (size-increase in specific gonadotropin-releasing hormone (GnRH) neurons in the brain), as well as an increase in GnRH release (Burmeister et al., 2005)). Other examples of social behavioural modulation studied from a molecular perspective include social feeding, sexual behaviour, social hierarchies, and dominance interactions (Robinson et al., 2005). In general, social influence on behaviour is modulated through communication - the emission (output), receiving (input), and integration of a social cue. This expands the scope of the mechanistic pathway in question from within one individual to between two or more.

The focus of my research has been to understand the genetic determinants of social interactions in *D. melanogaster*. The global hypothesis that has guided the studies reported here is that features of social interactions are inherited, and that the innate determinants of social interactions are associated with specific biochemical mechanisms and neural circuits in the central nervous system.
The thesis is organized as follows. **Chapter 1** is a review on social behaviour in *Drosophila melanogaster*. This chapter was originally published in 2012 as a review in *Advances in Genetics* as “One, two, and many – a perspective on what groups of *Drosophila melanogaster* can tell us about social dynamics.” Jonathan Schneider and I generated the first draft of the manuscript. This chapter provides background to illustrate what is known about the social life of the fly. **Chapter 2** is a more focused review I wrote for this dissertation. This chapter reviews the literature on circadian rhythms and social behaviour in *Drosophila*. **Chapter 3** was originally published in 2009 in *Nature* as “Specialized cells tag sexual and species identity in *Drosophila melanogaster*”. I am the second author on that paper. Together with Dr. Billeter, the first author, I collected data and I also worked on the revised manuscript. This paper highlights the importance of cuticular hydrocarbons in social signaling and recognition, and establishes methods and details that are used in subsequent chapters.

To be able to actually identify the genes underlying the pathways of social interaction effects, I took advantage of an established relationship between social interactions and biological time-keeping to address this hypothesis. Knowing the circadian rhythms of individuals can be affected by interacting conspecifics, I housed stimulus flies with focal flies expressing a luciferase reporter of circadian time. I used stimulus flies in forward and reverse screens to identify genes that affect social responses in *Drosophila melanogaster*. Stimulus flies in the forward screen were mutant fly lines generated by a chemical mutagenesis. Stimulus flies in the reverse screen evaluated candidate mutants. **Chapter 4** describes the mutagenesis screen as well as the methods I generated or edited for analyzing the outcomes from the screening assay. **Chapter 5** describes the results of the forward screen, while **Chapter 6** describes the methods and results used for the reverse genetic screen. Briefly, I measured the focal fly’s circadian pattern of luciferase expression over several days, and tested whether the stimulus flies altered this pattern when compared to focal flies maintained with controls. I also performed secondary screens involving known phenotypic aspects of circadian and social behaviour including locomotor activity assays, mating assays, and pheromonal profiling. I emphasize results for one candidate chemically mutagenized line and a *Drosophila* homolog of the *limbic system associated membrane protein* gene (*LSAMP*). **Chapter 7** concludes the thesis with discussions, overall conclusions, and future directions.
Chapter 1  ONE, TWO, AND MANY—A PERSPECTIVE ON WHAT GROUPS OF  
DROSOPHILA MELANOGASTER CAN TELL US ABOUT SOCIAL DYNAMICS


This chapter is an excerpt from a review on social behaviour in Drosophila melanogaster. It was originally published in 2012 in Advances in Genetics. Jonathan Schneider and I generated the first draft of the manuscript, whereby my efforts were focused on pages 59 to 70 of the published manuscript. Permission to reproduce this manuscript is included in APPENDIX A.V.1. It has been included here to provide a background on what is known about the social life of the fly, prior to presenting the studies I conducted to investigate the genetic determinants of social interactions.

1.1 ABSTRACT

In the natural world, interactions between individuals occur in groups: an individual must recognize others, identify social opportunities, and discriminate among these options to engage in an interactive behavior. The presence of the group is known to exert an influence on individual group members, and this influence may feedback through the individual to affect behavior across the group. Such feedback has been observed in Drosophila melanogaster, for example, when mating frequency increases in groups composed of mixed strains compared to homogenous groups (Billeter et al., 2012a; Krupp et al., 2008). A working hypothesis is that social processes—to recognize, identify, discriminate, and engage—are innate. They rely on a combination of genetic inheritance, molecular interactions, and cell circuitry that produce neural and immunological responses. Here, we discuss studies that emphasize social interactions in four categories in Drosophila melanogaster: learning, circadian clocks, aggression, and mating. We also speculate that a systems-level network approach to the study of Drosophila groups will be instrumental in understanding the genetic basis of emergent group-level behavior.

1.2 INTRODUCTION

The problem of tracing the emergence of multidimensional behavior from the genes is a challenge that may not become obsolete so soon (Benzer, 1971).
Although classified as a solitary insect species, *Drosophila melanogaster* displays a surprising array of behavioral interactions that are widely accepted as social: they court and mate; they fight over resources; they communicate using chemical, auditory, and tactile cues; they aggregate; they disperse; they synchronize their daily activity to one another; and there have been reports that they engage in observational learning to frame social expectations (Hall, 2002; Kravitz and Huber, 2003; Lefranc et al., 2001; Levine et al., 2002a; Mery et al., 2009; Sarin and Dukas, 2009; Suh et al., 2004).

When a behavioral assay involves only two flies, such as with assays of courtship and copulation, there is no choice about interactive partners. The decision to interact, or not, depends on the qualities and state of both interactee and interacter. The interaction itself is determined by the two individuals, and any behaviors exhibited by the dyad are modulations of their interaction. However, once in a group, the presence of many individuals produces a complex social context, and it becomes necessary for an interacter to connect with one among many potential interactees. Understanding patterns of copulation or any social interaction within the group may involve more than scaling up from one or two to many because the other members of the group form an environment that can modulate, facilitate, or disrupt the dyad (e.g., competition for a mate can preclude an individual’s copulatory behavior).

In addition to affecting patterns of behavior, the group also influences its individual members directly. This group influence may affect gene transcription, neuronal morphology, pheromonal communication, and subsequent behavior (Billeter et al., 2009; Billeter et al., 2012a; Donlea et al., 2009; Eddison et al., 2011; Kent et al., 2008; Krupp et al., 2008): as the group members change behavioral patterns and pheromonal profiles, the character of the social milieu can change (no detailed study of dynamics, but see (Kent et al., 2008)). In this way, the variety of behaviors that are known to be influenced by, and exert an influence on, the social context create complex systems with many interactions and feedback loops between individuals. The dynamics of such complex systems not only modulate an individual’s behavior and gene expression (see indirect genetic effects; (Moore et al., 1997), but such dynamics may also lead to emergent, group-level behavioral phenotypes.

The challenge of investigating group dynamics is therefore one of dissecting the many effects that co-occur within, and potentially modulate, the group. At a minimum, three social factors exert effects on behaviors within the group: number of potential interacters, genotypic composition, and timing of social experience. In a homogeneous setting composed of individuals from the same strain or
genotype, manipulating the number of individuals in the group can reveal nonlinear behavioral response curves and threshold values for social, group-level effects (Saltz and Foley, 2011; Wang and Anderson, 2010). Mixing together groups can uncover emergent dynamics that are not seen in the respective homogeneous settings and may uncover group-level responses to strain or species recognition and competition (Kent et al., 2008; Krupp et al., 2008; Levine et al., 2002a). The influence of the timing of the social context, whether prior to or during observation, can suggest how this change in the social surrounding is being integrated, via developmental changes, learning, or circadian clocks, for example.

The group can therefore be seen as a complex system that may give rise to the emergence of group-level behavioral patterns. While the cues underlying group behaviors continue to be discovered (such as sex and species “tags” (Billeter et al., 2009)), the mechanisms of these complex systems—the organization, how the flies assemble and disseminate information—are still a black box. If the rules of group organization are coded genetically, the difficulty in dissecting the path from gene transcription through behavior to group organization prompts the question asked by Michael Bate in another context, “[..] how [are] we going to look for the genes that regulate [social] behavior?” (Bate, 1998). Bate 1998 was asking about the genetic substrate required for a maggot to develop the ability to crawl; here, we are asking about the genetic requirements for participation in complex social groups.

In the first part of this perspective, we consider several broad behavioral categories and emphasize effects elicited by social context. We also present several cases of groups exhibiting emergent properties arising through the dynamic interactions of group members. While the investigation of such group-level phenotypes is not straightforward, their identification is becoming more and more frequent and can be done without specialized techniques.

In the second part, we advocate treating groups of flies as complex systems, with many interactions and feedback loops between individuals. These relationships may explain the emergence of group-level behavioral patterns and allow us to understand the mechanisms that generate them. We have begun to approach the biology of the fly at a group level using system-level tools from network theory. We speculate that such a systems-level approach to the study of Drosophila will be instrumental in answering our version of Bate’s dilemma.
1.3 Caveat

We have elected to write this as a perspective piece. We focus on *D. melanogaster* with the qualification that the analysis of group dynamics is warranted and carried out in other animals as well. There are many important studies that will not be presented here. We hope the authors of those studies—many of whom are friends—will not feel slighted.

1.4 The Behavioral Effects Of Social Context

1.4.a Social Learning And Memory

Whether an experiment relies on prior experience such as previous social contexts, competitive outcomes in aggression, or matings, the possibility of a social manipulation becoming a “conditioning” stimulus must be considered (Mery et al., 2009; Sarin and Dukas, 2009). Although untested, molecular and cellular mechanisms of learning and memory could prove informative when investigating the pathways underlying social interactions (Ganguly-Fitzgerald et al., 2006). Only a few studies have assessed the role of known learning substrates on social experience, yet the ability to learn and modify behavior as a consequence is likely to be a relevant feature of social experience. It is possible that learning pathways contribute to mechanisms of social engagement.

*D. melanogaster* has contributed insights into the genetics and neurobiology of learning. Many of these contributions were developed using olfactory learning assays such as the T-maze which rely on an index of learning and memory measured in groups (upward of 60 flies), but this feature of learning assays has not been a focus of study (Davis, 2004; Kahsai and Zars, 2011; Keene and Waddell, 2007; McGuire et al., 2005). It is usually assumed—and why not?—that mechanisms of learning, especially learning of an operant task, apply to the individual. Here, we restrict our discussion to studies that directly evaluate the influence of the social environment on learning and memory of the individual.

Several studies have reported that individual flies display the ability to change their behavior by observing other flies within a group, a form of social learning. For instance, whereas a female will remain in closer proximity to a healthy male in comparison to a poorly fed male, she will change her preference if she detects another female’s proximity to the unhealthy male (Mery et al., 2009). This demonstrates that females acquire information by observing others and display selective responsiveness to this input. The ability to acquire such information from the social environment is also evident when females display mating preferences for males that are dusted with the same color
as males previously observed copulating (Mery et al., 2009). This suggests that individuals are able to categorize socially acquired stimuli and remember them for subsequent mating choices. Selecting an appropriate egg-laying site is yet another process in which females exploit information provided by conspecifics (Sarin and Dukas, 2009). Individual females prefer food types as an egg-laying medium after they have experienced the food with mated but not virgin females. Moreover, a location preference for oviposition can be communicated without demonstrating egg laying on the medium itself (Battesti et al., 2012), indicating that naive individuals can recognize and learn from females who have been conditioned to prefer a particular egg-laying substrate. These studies demonstrate learned behavior based on the selective transfer of information between the individuals.

Despite such evidence supporting the idea that acquiring information from the social environment influences an individual’s behavior in a group, the possibility that classical learning pathways explain this influence has not been well tested. One study has examined the influence of conspecifics using a classical olfactory learning and memory retrieval paradigm (Chabaud et al., 2009). In this assay, flies are trained to avoid an odor using shock and then subsequently evaluated on the avoidance of that odor to assess anesthesia-resistant memory (ARM) formation and retrieval. Flies trained in groups or in isolation were tested individually, and no differences were observed. However, subsequent testing showed that flies tested in groups achieve higher scores than flies tested individually on the ARM task. This improved performance does not involve nonspecific aggregation or herding because it is observed even when the accompanying conspecifics have been trained on different odors. In other words, the simple presence of conspecifics during ARM retrieval seems to improve performance. The aspect of the social environment that facilitates this memory retrieval is still unknown and deserves further research. Yet, these findings clearly demonstrate that the social environment influences ARM-dependent performance.

More generally, these studies raise questions such as: When groups of flies are trained how many of the flies are conditioned by the experimental stimulus, how many are following the lead of others, how many acquire conditioning via social interactions within the assay, and how much of their performance depends on a social contribution to retrieval? When group conditioning is tested, is it a sum of individual information? If information is distributed throughout the group, is there positive feedback between individual and group information? Data suggest that when individual appraisals contradict public information, individuals do not always ignore what they have learned (Chabaud et al., 2009; Mery et al., 2009). On the other hand, agreement with others in the group is not necessary for the
socially mediated performance improvement in conditioning tasks (Chabaud et al., 2009). This complex interplay between a fly’s individual experience, social communication of information, a group’s conditioned status, and the influence of the group on the individual are all potentially at play when groups are investigated. The propagation of conditioning (Battesti et al., 2012) and facilitation of retrieval (Chabaud et al., 2009) could play a role in emergent group-level phenotypes (see (Billeter et al., 2012a; Higgins et al., 2005; Schneider et al., 2012b)).

1.4.b SOCIAL SYNCHRONIZING OF ACTIVITY AND REST

Flies aggregate at dawn and dusk in the wild (Shorrocks, 1972; Wertheim et al., 2005). The temporal organization of aggregation sets the stage for other social interactions, and it is based on circadian clock mechanisms in the adult brain. These clocks are thought to gate sensory excitability and enable an individual to anticipate key events in the environment. Individuals must be active, at the same time, in order to aggregate around food and to reproduce. Similarly, they must time their rest to ensure participation in group functions. Such biological synchrony can be coordinated by environmental cues such as the photoperiod, and the influence of the physical environment on the periodicity of the biological clock is well studied (Allada et al., 2001). Social synchrony might also be facilitated by the influence of social cues, but understanding the influence of social cues on the biological clock is just beginning.

Several studies have established that social groups influence the timing of various behaviors in flies, from locomotor activity bouts to circadian patterns of mating (Billeter et al., 2012a; Fujii et al., 2007; Krupp et al., 2008; Levine et al., 2002a; Lone and Sharma, 2011b; Tauber et al., 2003). Rhythmic patterns of behavior are coordinated within a group; flies maintained in groups are more synchronous in their individual patterns of activity than isolated flies under the same light:dark regimes (Levine et al., 2002a). This modulation of individual activity depends on the rhythmicity of flies in the groups and the ratio with which they are mixed. Arrhythmic mutants decrease synchrony among wild-type (WT) flies, and the strongest effects are seen on a rhythmic majority housed with an arrhythmic minority (Levine et al., 2002a; Lone and Sharma, 2011b). These effects are mediated by unidentified olfactory cues (Levine et al., 2002a; Lone and Sharma, 2011b). Temporal patterns of reproductive interactions are modified between cohabiting males and females (Fujii et al., 2007). Same sex couples remain synchronous to isolated individuals, but male–female couples reset the timing of activity when housed together (Fujii et al., 2007). Further, when groups of six males are maintained with six females, the
temporal pattern of mating is regulated by the strain of the female: the female’s pattern is evident whether placed with males of the same or different strains (Billeter et al., 2012a).

Although these studies suggest that flies may reset the clocks of other flies within a group, they do not prove that the clock mechanism is affected by the social environment. However, a subsequent study has shown that when groups are mixed in a way that is similar to the manipulation performed by (Levine et al., 2002a), the expression levels and amplitude of circadian clock genes in the head and in peripheral tissue respond to social manipulation (Krupp et al., 2008). These findings demonstrate that an individual’s circadian clock mechanism can be influenced by the social environment and suggest that synchrony is reflected in the transcriptional fluctuations that keep time. As noted, effects of these social manipulations on activity patterns depend on olfactory cues (Levine et al., 2002a; Lone and Sharma, 2011b) and require intact rhythmic sensitivity of peripheral clock cells in the affected group members (Krupp et al., 2008; Levine et al., 2002a).

A similar experimental approach demonstrated that social experience modifies individual sleep architecture, where the size of the group in which individuals are kept regulates the length of sleep bouts during the day (Ganguly-Fitzgerald et al., 2006). This plasticity in sleep requires intact visual and olfactory communication between the group members. In these studies, a variety of genetic, molecular, and neural components have been associated with the group effect. Whole brain dopamine levels as well as the expression of a subset of short- and long-term memory genes correlate with this social plasticity in sleep (Ganguly-Fitzgerald et al., 2006). Also, the expression of rutabaga, period, and blistered in a subset of circadian clock neurons is required for the display of experience-dependent variation in sleep architecture (Donlea et al., 2009). Interestingly, the morphology of these clock neurons is also altered: an increase in the number of synaptic terminals correlates with the social experience that influences sleep (Donlea et al., 2009). Thus, prior social experience influences circadian patterns of activity (Levine et al., 2002a; Lone and Sharma, 2011b) and sleep (Ganguly-Fitzgerald et al., 2006), neuronal morphology of clock cells in the brain (Donlea et al., 2009) as well as patterns of gene expression associated with timekeeping (Krupp et al., 2008). Taken together, these studies indicate that biological clock mechanisms not only regulate circadian behavior but are also modified by social stimuli.

These effects of the social group are not restricted to timing. Individual activity levels remain continuously susceptible to a variety of group properties such as genotype and gender (Higgins et al.,
In one study, a complex metric was formed from measures of many behaviors such as walking, grooming, feeding, mating, courting, and fighting (Higgins et al., 2005). This metric was used to determine the repeatability of intra-individual variation in isogenic groups using a repeated measures design. This study demonstrated that emergent characteristics of activity at the group-level explained as much variation in overall activity as the genotype of the group. However, the interpretation of this study is not straightforward because the “activity” metric itself is complicated. This metric does not discriminate between locomotion, sleep, and mating like the aforementioned studies. Instead, it reflects interplay between many or all behaviors and individuals of the group. It is tempting to ascribe such an emergent property as arising from the excitation and positive feedback of individuals interacting with one another. This study by Higgins et al., (2005) is especially noteworthy because the group-level activity is not easily predicted from the behavior of the individual members or from their genotype but from the ongoing interactions throughout the group.

Social groups can therefore influence patterns of behavioral activity by modulating the temporal structure of group interactions on a daily scale and within a given short-term sequence of events (Higgins et al., 2005). Notably, this organization of behavior influenced by the group provides a glimpse of an emergent property based on complex patterns of behavioral activity. Further, variable patterns of behavioral activity are evident even when the genetic substrate has been isogenized, suggesting that such emergent properties have a stochastic component that operates separately from the genetic component.

1.4.c Aggression

When flies meet, they may engage in a variety of offensive and defensive physical interactions, and highly detailed ethograms of these exchanges have been generated (Chen et al., 2002). Aggression in flies was thought to be a competition for resources, and original studies on aggressive behavior were performed in a group setting (Dow and Schilcher, 1975; Hoffmann, 1987a, b, 1988). Aggressive displays between pairs of males in groups were described as contests with a clear winner and loser. The underlying interpretation of early studies on fly aggression suggested the presence of a hierarchical group structure (Dow and Schilcher, 1975). The benefit of aggressive contests was thought to be establishing a territorial priority over food patches in the presence of other males for access to females (Hoffmann, 1987a).
There is considerable phenotypic variation in this behavior, some of which is the result of genetic variation (Hoffmann, 1988), body size (Hoffmann, 1987b), and age (Hoffmann, 1990). Given the inherent social aspect of aggression (needing at least one other fly to aggress), it is perhaps not surprising that prior social experience also plays a prominent role in aggressive behavior. Rearing individuals in the presence of males and not females significantly reduces aggressive behavior (Hoffmann, 1990). This adjustment is not simply the “loser effect” (experienced losers mostly lose second fights (Yurkovic et al., 2006)) of previous competitors, as individuals’ aggression toward males with which they have been previously housed does not significantly differ from that displayed toward unfamiliar males (Hoffmann, 1990).

Subsequent studies of aggression have undergone a shift away from observing aggression within a group to studying aggression between isolated pairs of males. Consequently, efforts were allocated to establishing a simplified yet optimized paradigm in which a pair of males would fight. This allowed rapid advancement in the identification of the genetics and neurobiology (see (Chan and Kravitz, 2007; Dierick, 2008; Dierick and Greenspan, 2006, 2007; Miczek et al., 2007; Robin et al., 2007).

Despite the great strides made in studying aggression, the current paradigm shift away from the group has hindered the analysis of aggression’s influence on social organization (specifically the hierarchy (Dow and Schilcher, 1975)). Unfortunately, studies investigating the “loser effect” are limited to a two-fight paradigm. This raises several questions. Is the “loser effect” cumulative? Can it be mitigated/erased by a victory? If the decrease in aggression and probability of winning is proportional to the amount of loses, a hierarchy seems inevitable. Even if winning mitigates the “loser effect,” since flies appear to have individual as well as general history-dependent aggression, a loser–winner may still reduce the aggression toward his initial opponent, again creating a hierarchy, although one in which the dynamics of contest outcomes becomes pivotal. However, to address such hypotheses, the current aggression paradigm must expand past the two-fight stage, to three bouts (at a minimum) or a group (optimal).

Current studies are beginning to bridge the gap between dyadic aggressive contests and group-influenced aggression. One aspect of group-influenced aggression, the olfactory contributions of a group, has been shown to stimulate male–male aggression (Wang and Anderson, 2010). Wang and Anderson, (2010) posit that cis- vaccenyl-acetate (cVA) cue may play a dual role: not only is it a well-studied aggregation pheromone (Wertheim et al., 2005), but it may be also acting as a dispersal cue
when groups reach critical mass through aggression and territoriality. Wang and Anderson’s (2010) model is parsimonious with regard to the independent finding of highly aggressive strains forming less dense groups (Saltz and Foley, 2011) and predicts that group density and group-level aggressiveness are part of a negative feedback mechanism linking group structure to individual behavior. This suggests that aggression may reduce the amount of cVA required to cause dispersal. However, cVA performs many functions, some inconsistent with others, a noteworthy topic beyond the scope of this perspective.

A full model of group-level aggression may therefore shed light on group composition. However, any such model must incorporate both the dynamic effect of aggression on density and the experience-dependent modulation of individual aggressiveness. Flies learn from and remember previous encounters (Yurkovic et al., 2006), and the aggression of an individual at a given point in time is influenced by a complicated result of not only its previous conflicts but also the opponent’s previous aggressive bouts (the results of which themselves are influenced by past opponents). While the depth of history that affects the current aggressiveness of a fly is still unclear, it is tempting to visualize the propagation of “wins” and “losses” as a network of aggressive bouts. The size of the group, the pattern, and outcome of these bouts affect not only an individual’s propensity to aggress but also the density of the surrounding group.

This dynamic balance between winners and losers and the modulation of group density is complex. Quantifying the properties of such an aggressive network would allow the prediction of whether group-level aggression approaches a genetically determined equilibrium, or if a stochastic process takes shape (the former similar to examples below (Billeter et al., 2012a; Schneider et al., 2012b) and the latter similar to the group-level activity reported by (Higgins et al., 2005)).

1.4.d Mating, Paternity, and Offspring

We have discussed the social influences on learning, on synchronizing patterns of activity, and on aggression. These socially modulated behaviors also seem to contribute to reproductive decisions in Drosophila. Although courtship effort can also be considered as a component of fitness, we restrict our discussion to the simpler and more fitness-related measurements of copulation and paternity. Prior social experience has significant input on female mate choice (Mery et al., 2009). Females prefer males of a specific color after observing males of that color copulate, and males colored differently get rejected. We also note a circadian aspect of mating, with WT flies exhibiting a cyclical mating rhythm.
under the control of the same clock genes underlying locomotor activity rhythms (Sakai et al., 2002). Yet, even when in the same aggregate at the same time, a male must obtain a female despite conspecific male competitors. This competition is thought to involve aggression which has been tied to copulation, as the presence of a female increases aggressiveness, and winning is correlated to success in mating through food territoriality (Dow and Schilcher, 1975; Hoffmann, 1987b).

Earlier studies point to evidence that females are also able to distinguish between the minority and the majority in heterogeneous groups of males through olfactory cues; these early studies dubbed this a “rare male effect” as preference was displayed toward the minority males (Ehrman, 1970, 1972). Recently, this idea of heterogeneous group effects on mating behavior was revisited. Within a 24-h period, the males in a genotypically homogeneous group mate fewer times than the males in a heterogeneous group (Billeter et al., 2012a; Krupp et al., 2008). There is also a parallel influence of group composition on individual physiology (Kent et al., 2008; Krupp et al., 2008) and on the expression of clock genes as well as on the expression of genes involved in pheromone synthesis (Krupp et al., 2008). These changes in gene expression are accompanied by changes in the production of key hydrocarbons, which in turn are known to play a major role in communication and reproductive behavior (Billeter et al., 2009). The reproductive consequences associated with changes in hydrocarbons are further supported by studies where male group heterogeneity does not increase male matings when housed with olfactory mutant females and also indicates that this social effect is at least partly female driven (Billeter et al., 2012a).

A similar effect is observed when females of one strain are placed with a genotypically heterogeneous mix of males at varying ratios (Billeter et al., 2012a). Male mating success and female fecundity are affected by the interaction of male strain and group composition, and both are dependent on female strain. Surprisingly, when examining all offspring produced by the group, each male strain sires a consistent percentage of all offspring regardless of the ratio of heterogeneity in the group. This is particularly interesting given that this phenotype arises as a group property and cannot be detected in any one female within the group. This illustrates that while individual behavior can be variably responsive to the social environment, it can do so in a controlled fashion that can generate a consistent group phenotype. The mechanistic underpinnings of such regulation are still unknown. However, one possibility lies in the manipulation of last male sperm precedence as any individual female can potentially negate the effect of last male sperm precedence when copulating with multiple individuals (Singh et al., 2002). Given unrestricted access to males in a group, a dilution effect is seen concerning
the paternity patterns, and it is possible that females could modify the paternity according to the order, and the amount of copulations (Billeter et al., 2012a). While this possibly describes how a female fly modifies her offspring ratio, it does not address the mechanisms of the evaluation of and response to the social context. Are the pathways involved in evaluating the social context restricted to olfaction? Is the female quantifying the masculine environment? What pathways are involved in the female’s subsequent predisposition to mating with a certain male? Does the male exhibit any influence through seminal fluid transfer? Are females observing conspecific matings to learn the patterns of copulation of the group? It is also noteworthy that this is only observed in one strain, while the other strain studied simply maintains an elevated frequency of mating in any social environment. This elevation does not produce a fixed ratio of offspring but may increase offspring genetic variability by indiscriminately increasing the number of matings. This strain-dependent difference in female reproductive strategies may eventually shed light on the genetic complications associated with the rare male effect, which is more prominent in certain strains than others, and may highlight shared mechanisms underlying strain-specific reproductive decisions.

Given the emergent property of offspring genetic diversity, it is unlikely that these reproductive decisions are based solely on an individual’s chemical sampling of the heterogeneity of the group. Rather, we hypothesize that the process is a dynamic one, determined not only by a female’s copulatory history but also by the prior and ongoing copulations within the social context. This process may be affecting both males and females and may be partly explained by seminal fluid transfer: not only is any male being modulated by the presence of potential conspecific rivals (Bretman et al., 2009), but the female’s physiology/behavior could also be modified, which would in turn affect future copulations/sperm transfer (Luepold et al., 2011). In tandem, a female’s observation of her conspecific’s choice in mating partners may affect her predisposition to mate with a given type of male. Investigations into these potential processes require an approach that incorporates the current, as well as the past, patterns of copulation as this process takes shape. These complex histories of prior mating experience, visual conspecific learning, physiological changes, all potentially play a role, and deciphering the patterns of the group will be pivotal in investigating the group-level mating phenotype and its consequences.

Although the emphasis on group dynamics is relatively new, several themes are already emerging. For example, the social environment affects gene expression and metabolism within individuals. Additionally, interactive behaviors, such as the frequency or temporal distribution of mating, vary
when the group is manipulated. The implications of such variability in an interactive behavior may not be trivial. Moreover, the distinction between an individual and its environment may be a useful experimental device, but it too could be artificially limiting. We have been developing a method to examine the group as a social interaction network, a complex group-level entity. Our results suggest the possibility that traditional social behaviors—like mating and fighting—are influenced by the details of an underlying social interaction network.

1.5 CONCLUSION

The complexity and challenge of evaluating group-influenced behavior arises because observing a specific behavior necessarily involves consideration of other behaviors—how do locomotion and aggression influence sleep, for example? Within a group, behavior of an individual influenced by his social environment feeds back onto the group, which in turn might readjust its influence. Once this concept is applied to the variety of behaviors that are known to be influenced by the social context, many of which occur simultaneously, we begin to see that groups of Drosophila can be considered complex systems with many interactions and feedback loops between individuals. The group is a complex system that may give rise to the emergence of group-level behavioral patterns.

The discovery of emergent group-level phenomena also shows that a group is a complex system with phenotypes that could not have been accurately predicted by simply “scaling-up” dyadic interactions. Rather, robust emergent properties are seen. This is intuitive as these behaviors did not evolve in isolation; they occur and were selected within, and interact with, the social environment. In other words, we will not be able to really understand the neurobiology of behaviors such as courtship, mating, and aggression until we can account for the role of the group.

When a phenotype (including a group-level one) is robust and depends on a genetic component, the question becomes “how?” What is happening in the group that facilitates ARM retrieval? How does the “group” explain as much variation in behavior as the genetic component? How does the mixing of two strains of males produce a remarkable fixed split in the paternity of the offspring independent of the ratio of the males? How does this pattern of offspring diversity arise even when any individual female does not produce this paternity ratio? We are forced to conclude that certain properties of the group are not simply properties of individuals or dyads “scaled-up” in number.
How does an individual’s genotype contribute to emergent phenomena at the group level? How does the group interact with an individual’s genetic composition? We are reminded of the similar paradox discussed by Bate; whereas he discussed the genetic contribution to neurons that interacted and associated to create emergent, organism-level behavior (Bate, 1998)), we consider the genetic contribution to individuals, associating and interacting in a social space to create group-level phenomena. In exploring this paradigm, we come to the question, as did Bate, of how is it possible that the complexity of the group can be coded in the genetic composition of the individual? Is the complexity a “global dynamical system with many interactions” or rather “defined subprograms that [organisms] can get hold of and execute for themselves” (Brenner, 1974)?

Given the potential complexity of the social environment of Drosophila in the wild (variations in sex ratios, number of conspecifics, amount, and number of various hetero-specifics (Shorrocks, 1972)), it becomes improbable that flies have their behavioral outputs (social “roles” or responses) hard-coded to respond to their social context. This suggests that the interactions throughout the group regulate individual behavior in a dynamic fashion to form a cohesive group effect.

These “interactions” in theory are any and all interactions between one or more flies. However, one could hypothesize that interactions which feedback to regulate social behavior would themselves be affected by social context, allowing a much more dynamic process of group interaction. This modulation of robust, innate behaviors could potentially explain the propagation of effects and stabilization of a group: each fly’s social behavior has been modulated by its own history and is being modulated by its surroundings. In a dynamic system, exploring a single propagation of effect among many seems daunting. In theory, one should be able to attempt to discern the patterns that result from the behavioral interactions, and how these are organized to establish group cohesion (cohesion in the sense required for a robust group-level effect).

The ability to understand the group, therefore, appears to be linked with the ability not only to understand how each behavior is modulated by social context (of which much has been shown) but also to understand how these behaviors are patterned throughout the group, how these complex patterns arise from individuals, and eventually how this information is contained in the genome. One approach is to study the social group on a systems-level basis via network analysis. This is not a novel approach to group dynamics and has a long history of successful insights into the social organization of humans, monkeys, dolphins, and ants among others (Croft, 2008; Formica et al., 2012).
Hopefully, the genetic dissection of patterns that may underlie emergent group phenotypes will help us understand the complexity of a group. However, this network investigation cannot exist without continued experimentation of social behaviors in other contexts. Dyadic interactions currently allow greater detail and throughput, and offer promise to continue to shed light on the implications of social interactions for the individual, yet they are simply one part of the continuum of social and groups. We are not simply asking about one versus two versus many, we are asking about one among many and many among more. Using a multilevel approach across disciplines, we may be able to understand the social life in more-than-minimal groups of *D. melanogaster*, the insect formerly known as “solitary.”
Chapter 2 MECHANISMS OF SOCIAL INFLUENCE ON D. MELANOGASTER LOCOMOTOR ACTIVITY

Locomotor activity is a generic behavioural output that has been studied extensively (Dubruille and Emery, 2008; Hardin, 2005; Stanewsky, 2002). The term “generic” is used here, as little is known about which behavioural outputs general activity represents in D. melanogaster (De et al., 2013). It is known, however, that locomotor activity is highly responsive to the social environment (Dubruille and Emery, 2008; Levine et al., 2002a), which implies that changes in generic activity may reflect the coordination of behaviour(s) within the group. Notably, a number of studies investigating social influence on locomotor activity have identified underlying genetic and neuronal substrates (Fujii and Amrein, 2010; Fujii et al., 2007; Krupp et al., 2008; Levine et al., 2002a). The importance of these findings is far-reaching and does not only relate to the mechanistic understanding of the activity modulation by the social environment. Since activity encompasses other behaviours, these mechanisms might represent common substrates with which an individual adjusts other behaviour(s) in response to the social surroundings. This perspective is supported by several studies that have highlighted the involvement of the clock system in mediating the effect of social experience on locomotor activity (Fujii et al., 2007; Levine et al., 2002a), as well as other behaviours such as courtship (Fujii and Amrein, 2010; Fujii et al., 2007) and mating frequency (Krupp et al., 2008). It is thus fair to posit that research investigating the social regulation of activity at the mechanistic level might prove informative in investigating the substrates involved in the social regulation of other behaviours.

This chapter reviews genetic and neuronal substrates through which the social influence on activity is mediated. Several features of the social environment are addressed, including gender, group size, and group composition (Table 2-1). The timing, duration, and frequency of social manipulations are also considered, as well as whether the resulting influence on activity is manifested immediately and/or is long lasting. The identified genetic and neuronal substrates involved in the generation and integration of social signals will provide the background for subsequent investigations carried out and described in this dissertation (Chapter 4, Chapter 5, and Chapter 6).

Given that locomotor activity is clock-controlled, background regarding the time-keeping system in D. melanogaster is first provided below.
2.1 *D. melanogaster* Clocks

Biological clocks are endogenous molecular pacemakers that oscillate to control the timing of cell biochemistry, body physiology, and behaviour (reviewed in Dubruille and Emery, 2008; Hardin, 2005; Stanewsky, 2002; Hermann-Luibl and Helfrich-Förster, 2014; Hardin and Panda, 2013). These clocks evolved as an adaptation to varying physical cycles, such as light and temperature, and to changes in the ecological environment, such as the activity of prey or predator. In *D. melanogaster*, the time-keeping system is well understood on the molecular and cellular level (Dubruille and Emery, 2008; Hardin, 2005; Hardin and Panda, 2013; Stanewsky, 2002), and numerous behaviours with varying rhythmicity have proven to be under the control of biological clocks. The most extensively studied outputs are those characterized by circadian rhythmicity, including eclosion (Myers et al., 2003), locomotor activity (Dissel et al., 2014; Dubruille and Emery, 2008; Hardin, 2005; Hermann-Luibl and Helfrich-Förster, 2014; Stanewsky, 2002), courtship (Fujii and Amrein, 2010; Fujii et al., 2007; Hanafusa et al., 2013), and olfactory sensitivity (Krishnan et al., 1999).

The circadian time-keeping system of *D. melanogaster* consists of clock cells found in the brain and numerous organs, such as the eyes, antennae, wings, oenocytes, forelegs, guts, and Malpighian tubules. These cells contain a circadian pacemaker composed of a self-sustained negative transcriptional feedback loop that oscillates with a period of around 24 hours (reviewed in Hardin and Panda, 2013). Within this loop, CLOCK (CLK) and CYCLE (CYC) heterodimerize and bind to the promoter regions of *per* and *timeless* (*tim*), thereby increasing their expression. PER and TIM in turn heterodimerize in the cytoplasm, where TIM protects PER from proteosomal degradation. The heterodimer complex then enters the nucleus and inhibits the activity of CLK/CYC. The timing of PER/TIM’s entry into the nucleus depends on several post-translational modifications that are also heavily regulated. Also, two additional interlocked feedback loops play roles in the *per/tim* and *Clk* transcription (Hardin, 2005; Hardin and Panda, 2013; Stanewsky, 2002).

In the brain of *D. melanogaster*, clock genes are expressed in numerous neuronal groups. Various sets of these neurons have been linked to different aspects of circadian rhythms (Dubruille and Emery, 2008). For instance, a group of neurons known as the Morning-cells (M-cells) are necessary for free-running locomotor activity in constant darkness. It has been noted that the fruit fly’s circadian pacemaker in the brain and other tissues are extremely sensitive to light, and these tissues can be entrained autonomously when cultured (Plautz et al., 1997). The molecular component that feeds light
cues into the oscillator in most of these tissues is the blue light intracellular photoreceptor CRY (Stanewsky et al., 1998). When activated by light, CRY binds to TIM and induces its degradation. The dissociation of TIM from PER in turn causes the proteosomal degradation of PER. This resets the intracellular pacemaker and synchronizes the clock in most tissues of the fly, including the M-cells. Other environmental cues, such as temperature and social interactions, also affect the pacemaker behaviour (reviewed in Dubruille and Emery, 2008; Hardin, 2005; Stanewsky, 2002; Hermann-Luibl and Helfrich-Förster, 2014; Hardin and Panda, 2013).

2.2  D. MELANOGASTER LOCOMOTOR ACTIVITY

Male and female D. melanogaster locomotor activity is rhythmic and dependent on abiotic factors, such as photoperiod, temperature, and food (reviewed in Dubruille and Emery, 2008; Hardin, 2005, Hardin and Panda, 2013). Single male locomotor activity rhythm (SLR) is characterized by a morning and evening peak in a twelve-hour light–dark cycle. The neural circuity that produces this bi-modal pattern has been well studied. Briefly, an intact circadian oscillator in the fifth small ventral and dorsal lateral neurons and possibly a subset of the dorsal neurons (5\textsuperscript{th} sLNv, sLNds, and DN1s) is sufficient for the evening peak, whereas the sLNv oscillator is sufficient for the morning peak. SLR is sustained in constant darkness (DD), albeit with an evening peak only; thus, the sLNv oscillator is sufficient for SLR in DD. This model has been significantly advanced in the past years to reveal more complex interactions between neuronal groups underlying activity patterns under different conditions (Dissel et al., 2014; Yao and Shafer, 2014). It is thus more accurate to state that circadian activity patterns are an emergent phenotype. It has also been shown that SLR is gender-specific: female activity is rhythmic but contains an evening peak only (Fujii et al., 2007), as females rest much less during the day (Dubruille and Emery, 2008). Activity patterns of males and females are not only gender-specific and dependent on abiotic factors, but also show immediate and long-term responses to the social environment (Frenkel and Ceriani, 2011).

2.3  IMMEDIATE RESPONSES TO THE SOCIAL ENVIRONMENT

2.3.a  PAIRWISE GENDER-SPECIFIC ACTIVITY AND CLOSE-PROXIMITY RHYTHMS IN DD

The activity pattern of isolated males differs from males housed with a conspecific. In a male-female setting (MF) setting, velocity patterns interpreted as activity are characterized by a trough at anticipated dusk and high activity throughout the subjective night (Fujii et al., 2007). This pattern not
only differs from that of solitary males, but is also dissimilar to that observed in a male-male (MM) setting. This indicates that a male’s social response to companionship is gender-specific. It is noteworthy that males removed from MF pairing revert to basic SLR in DD, indicating that MF rhythm is not entrained and requires the constant presence of a female cohabitant.

This socially-triggered variation in activity pattern might be due to the expression of certain behaviours that would otherwise not occur in a solitary or MM setting. For instance, MF activity changes might be the result of sexual interactions. Indeed, MF activity patterns in light dark (LD) settings correlate with LD and DD close-proximity rhythms and courtship (Fujii et al., 2007).

The expression of courtship is a social response that requires the presence of a female acting as a ‘releaser’ (Tinbergen, 1990). This social influence however is more radical than the simple release of courtship behaviour. This becomes clear when the pattern of courtship is compared to a male’s activity state in isolation. The MF close-proximity peak occurs at times when a solitary male is least active, whereas the close-proximity trough occurs at times when a solitary male is most active. This implies that courtship is not manifested within the active ‘space’ determined by SLR. In other words, courtship does not strictly occur during periods when a male is active. Furthermore, additional activity attributed to courtship is not combined with SLR patterns in an additive manner. If this were the case, an increase in activity levels at certain or all times of the day would be observed. This suggests that the social environment has the ability to modulate and radically restructure the intensity and timing of activity levels. Since activity is clock-controlled, this raises the question as to whether such a strong social influence overrides SLR and imposes a pattern independent of the pre-existing male clock status; or whether the signal is integrated within the male’s clock mechanism to generate a response resulting from the coordination of both the social setting and the male’s individual state. Studies have pointed to the latter possibility, as the male’s clock seems to be a determining factor. Males and females entrained in different time zones and placed in an MF setting display close-proximity rhythms in DD characteristic of the male’s internal clock (Fujii et al., 2007). Furthermore, null (per^o) and short (per^s) period mutant males paired with WT females have arrhythmic and short-period close-proximity patterns in DD respectively (Fujii et al., 2007). Consequently, although the MF social context radically restructures a male’s activity patterns, it does so in a manner that is dependent on the male’s internal state. Coupled with the aforementioned association with courtship, this social context- and male-dependent close-proximity rhythm has been referred to as male sex-drive rhythm (MSDR).
Given that the male’s clock mechanism plays such a significant role in the manifestation of this social response, studies have focused on determining the role of different clock neurons in the occurrence of MSDR. One approach involved using a variety of \textit{gal4} drivers to drive expression of \textit{UAS-CYCD\textsubscript{\textDelta}}, a transgene that specifically blocks circadian molecular oscillations without affecting other neuronal functions (Fujii and Amrein, 2010). Through such manipulations (Table 2-2, Rows 10-20), it was found that either or both of the sLN\textsubscript{\textDelta}s and ILN\textsubscript{\textDelta}s, as well as the DN1s are necessary for MSDR. However, given the combination of manipulations considered, the role of other neuronal clusters cannot be eliminated. Furthermore, it is worth noting that there exists a driver-specific effect when manipulating the \textit{Pigment-dispersing factor (Pdf)}-positive LN\textsubscript{\textDelta}s. Disruption of molecular cycling using \textit{Pdf-gal4} does not affect MSDR, whereas using the seemingly more active driver \textit{Mz520-gal4} abolishes MSDR. In another approach, a variety of \textit{gal4} drivers were employed to drive \textit{per} expression in an otherwise \textit{per\textsuperscript{0}} background (Table 2-2, Rows 22-28) (Fujii and Amrein, 2010). Based on these manipulations and in combination with the above results, it becomes clear that male \textit{per} expression in the LN\textsubscript{\textDelta}s and DN1s is necessary and sufficient to produce MSDR in DD. It is not clear, however, whether either or both of the sLN\textsubscript{\textDelta}s and ILN\textsubscript{\textDelta}s are required. Another study has also aimed at investigating the role of different clock neurons in the manifestation of MSDR (Table 2-2, Rows 1-9) (Hamasaka et al., 2010). The design of this study however, makes a definitive interpretation of the results problematic for several reasons. First, both males and females were simultaneously manipulated. Although the male’s role has already been proven to be key in MSDR (Fujii et al., 2007), it would be premature to confidently deny potential effects emanating from the manipulated female. Second, important MSDR markers such activity dips at dusk were only compared descriptively without carrying out the necessary statistics. Furthermore, the combination of manipulations carried out is insufficient to confidently resolve the neuronal contribution to MSDR (Table 2, Rows 1-9). More specifically, the only conclusion that can be made is that one subset of the DN1s seems unnecessary for MSDR and the role of the rest of the clusters cannot be ruled out. Collectively, the available reliable data addressing the role of different clock neurons in the manifestation of MSDR, reveal that the LN\textsubscript{\textDelta}s and at least some DN1s seem necessary and sufficient to produce a WT MSDR in DD. Other clusters might also be involved, but further work is needed in that regard.

Studies have shown that the majority of clock neurons (sLN\textsubscript{\textDelta}s, LN\textsubscript{\textDelta}s, and some DN1 clusters) express \textit{fruitless (fru)} and are, therefore, physiologically sexually dimorphic (Fujii and Amrein, 2010; Stockinger et al., 2005). Such \textit{fru} mutants have normal SLR and PER cycling in clock neurons, as well as normal
courtship activity towards both males and females (Stockinger et al., 2005). However fru mutant males do not display rhythmicity in MSDR (Fujii and Amrein, 2010). When male-specific FRU (FRUM) expression is suppressed in clock neurons only, MSDR remains intact, indicating that FRUM might play a role in non-clock cells or that the tools employed might have been insufficient to completely suppress FRUM in these cells (Fujii and Amrein, 2010). Research in this area will be informative, as it will identify regulatory elements that interact with an individual’s clock to regulate behaviour. Also, due to fru’s role in the development of male circuitry, such investigations might present a developmental perspective to an individual’s response to the social environment.

Which sensory mechanisms mediate MSDR? Given that MSDR persists in DD, visual stimuli seem unnecessary for social influence on activity. However, studies have shown that the olfactory system is required for this social adjustment, as the removal of male antennae or the use of null mutant males for the broadly expressed Or83b olfactory-receptor gene (Or83b) abolishes MSDR (Fujii et al., 2007; Krishnan et al., 1999). The correspondence between the increase in male night activity in MF settings and the peaking of olfactory sensitivity during the night (Krishnan et al., 1999) suggests that female-derived chemosensory signals (among other potential signals) are detected by males’ enhanced nighttime olfaction, leading to an increase in courtship activity. It is interesting that despite potential hydrocarbon cycling (Krupp et al., 2008), MSDR follows the male’s circadian clock irrespective of the female’s clock status and consequent hydrocarbon profile. This suggests that, at least in the case of MSDR, a male responds to female hydrocarbons as a gender identifier rather than a signaling modulator for timing of reproductive behaviour.

Based on the available reliable studies discussed above, it may be concluded that the presence of the female is detected by the male through olfaction, triggering courtship behaviour. This female-induced courtship behaviour is not additively overlaid on SLR, does not operate within the boundaries of SLR, and does not override SLR irrespective of male clock status. Instead, the social signal is integrated within the male’s internal clock in the LNv and some or all DN1s to produce a male–female coordinated WT MSDR in DD. Other neuronal clusters might also be involved, but further work is needed in that regard.

2.3.b PAIRWISE GENDER-SPECIFIC ACTIVITY RHYTHMS IN LD

MF social interactions have been also investigated in the presence of light, a strong Zeitgeber (circadian coordination signal; ZT), albeit through a modified assay. Instead of measuring velocity or close-
proximity patterns of individuals in a pair, MF activity of couples was measured in standard locomotor activity tubes (Hanafusa et al., 2013; Lone and Sharma, 2012). The validity of this method was confirmed by comparing the activity of couples to the sum of 2 solitary individuals. Through this comparison, it was confirmed that the activity pattern of pairs is unique and is not a mere artifact of summing the activity of 2 individuals. Consistent with velocity patterns and MSDR (Fujii et al., 2007), MF couples are more active than MM pairs during night periods in LD. It is noteworthy that this nocturnality seems prevalent in several strains including Canton-S (CS), Oregon-R (OR), white$^{118}$ mutants, and yellow white (yw) mutants (Lone and Sharma, 2012). Thus, these findings reinforce the existence of a gender-specific social response.

The nocturnality observed in this assay has also been shown to be associated with courtship. When female-specific fruF splicing mutant males were paired with WT females, nocturnality was abolished (Hanafusa et al., 2013). Given that courtship towards females is suppressed in these males, it follows that nocturnality is strongly associated with courtship.

The mechanism underlying the social response to MF setting in LD has also been investigated in this modified assay (Hanafusa et al., 2013; Lone and Sharma, 2012). Notably, both males and females were manipulated simultaneously in most of the investigations mentioned below, as opposed to one gender at a time, as was performed in MSDR (Fujii and Amrein, 2010; Fujii et al., 2007). When per$^0$ MF were considered, nocturnality persisted. This suggests that in contrast with DD settings, intact circadian molecular oscillations are not required for the establishment of this behaviour in LD. Also of note, when males were removed from MF couplings, increase in nighttime activity was immediately abolished just as was observed in MSDR (Hanafusa et al., 2013).

Other aspects of D. melanogaster clocks have also been investigated in the context of nocturnality (Lone and Sharma, 2012). For example, in contradiction with DD MSDR, Pdfl mutant MF and individuals with electrically silenced LN,$s$ exhibit an increase in total nighttime activity in LD. These findings further support the notion that sexual interactions enhance nocturnality independent of the molecular clock in LD. From an objective point of view however, a closer look at activity patterns as opposed to considering totals in these latter manipulations, indicates that the increase is not as prominent as is found in WT MF. This would have been more evident in the totals if the sums were conducted over the more consistent middle-nighttime period and excluded the more variable activity patterns during day-night/night-day transitions. As a result, although PDF neurotransmission seems unnecessary for
this social response in LD, a closer look at the data suggests that these PDF neurons might play a role. Given this and the doubts regarding Pdf-gal4 vs Mz520-gal4, it would be preferable if both drivers, Pdf0 mutants, LN,s-specific suppressed clock gene expression, as well as individuals with silenced LN,s, are used in future studies before any conclusions can be reached as to the role of these neurons. The interpretation of PDF signaling is not the only finding that presents a challenge to the non-requirement of clocks in the establishment of nocturnality in LD. Silencing of CRYPTOCHROME (CRY)–positive clock neurons, for example, abolishes any increase in nighttime activity in LD (Lone and Sharma, 2012).

Although the collective attempts to investigate the mechanism underlying nocturnality in LD seem contradictory, a potential model might be at play. It has been noted that per0 mutant males suffer from a disrupted molecular oscillator in both LD and DD. This results in arrhythmic activity in DD, but their locomotor activity in LD is rescued due to extraocular photoreception (Hardin, 2005). As per0 males display almost normal nocturnality in LD and not DD, it may be that female input is able to modulate male locomotor activity and nocturnality irrespective of functioning circadian molecular oscillator rhythmicity in LD. Similarly, the circadian molecular oscillations of cry mutants are disrupted in LD, but locomotor rhythms remain intact as a result of extraocular photoreception (Stanewsky et al., 1998). The difference between the two lies in whether the affected circuitry is in a collectively coherent or unphased status. Although molecular oscillations in per0 and cry mutant males are disrupted, it is possible that the affected circuitry maintains a certain level of coherence in the former and not the latter. Since silencing of CRY-positive clock neurons abolishes nocturnality in LD, it can be hypothesized that nocturnality requires a certain level of circuitry coherence. On the other hand, it might also be possible that non-clock-related functions in the silenced CRY-positive neurons are necessary for nocturnality. Furthermore, both Pdf0 mutant MF as well as individuals with electrically silenced LN,s are characterized by impaired synchrony within the clock circuitry and suffer a decrease in LD nocturnality. These results hint that although an intact male circadian oscillator is not required for nocturnality in LD, clock coherence as well as non-clock-related functions in clock neurons might be necessary. This hypothesis, however, requires thorough investigation.

In terms of sensory modalities, it has been found that MF Or83b mutants do not display enhanced nocturnality in LD (Lone and Sharma, 2012). This is consistent with MSDR in DD and suggests that olfaction is necessary for the establishment of increased MF activity. A closer look at activity patterns in this study, however, as opposed to totals, shows that a partial activity increase persists in these anosmic mutants. This opens up the possibility of other sensory modalities or questions whether
olfaction is completely blocked in these mutants. This line of questioning is resolved upon taking a closer look at specific subsets of olfactory-receptor neurons (ORN). Specifically, the olfactory receptors encoding genes *Or65a, Or88a, Or47b,* and *Or67d* are thought to play a role in MF communication (van Naters and Carlson, 2007). Electrical silencing or ablation of *Or47b* ORNs completely abolishes MF increased activity, indicating that *Or47b* ORNs are indispensable for the regulation of this social response. Furthermore, the ablation of *Or65a* and *Or88a* ORNs does not completely block MF increase in activity, but significantly alters activity levels, suggesting that these ORNs may also play a role. Notably, the ablation of Or67d ORNs, as well as the ablation of gustatory receptor neuron Gr5a (Vosshall and Stocker, 2007), do not impact MF-specific activity patterns (Lone and Sharma, 2012).

A comparison of male and female contributions to MF activity patterns has also been tested (Lone and Sharma, 2012). In *Or47b*-ablated males in the presence of WT females (M-F+), the lowest nighttime activity levels were displayed in comparison with WT male and female pairs (M+F+) or WT male and *Or47b*-ablated female (M+F−) pairs. Based on these findings, and consistent with MSDR (Fujii et al., 2007), males play a key role in driving increased MF nighttime activity. It is worth noting that females might also play a role in this social response as M-F+ pairs have higher nighttime activity levels than *Or47b*-ablated male and female (M-F−) pairs. Further, if nighttime activity patterns are considered as opposed to totals, M+F− show a partial decrease in activity compared to M+F+, reinforcing the female role in this social response.

There is a gap in research to confirm and/or dissect the immediate activity response of males to MM setting in LD. One study reported that MM pairs displayed erratic rhythms compared to single males (Fujii et al., 2007) indicating the presence of a social response. Another study measured single males and MM pairs in locomotor activity tubes (Lone and Sharma, 2011b). Their results indicate that MM patterns do not differ from the corresponding mathematical control (summing two males), which negates the presence of a social influence. Using the same assay, a very similar pattern is evident from the investigations of another group (Hanafusa et al., 2013), although M versus MM setting were never directly compared. On the other hand, a social influence on MM activity patterns has been observed when a long period (*per*) mutant is paired with another individual (Lone and Sharma, 2011b). This is not observed with other *per* alleles, suggesting that such a social response might occur when an individual’s clock status is different in a directional manner. Needless to say, the immediate activity consequences of MM interactions, if any exist, require thorough investigation to resolve these contradictions.
Immediate activity response of females to female-female (FF) pairings in LD remains undeveloped. Two studies briefly include such data, both of which seem to indicate that activity of FF pairs does not differ from that of single females (Fujii et al., 2007; Hanafusa et al., 2013).

Collectively, the studies considered in this section indicate that MF couples in LD display increased nighttime activity levels as observed in DD, and that this increase is also associated with courtship. In contrast with DD settings, intact circadian molecular oscillations are not required for the establishment of this behaviour. Instead, clock coherence, regardless of rhythmicity, as well as non-clock-related functions in clock neurons might be necessary for this nocturnality. Consistent with DD settings, olfaction is necessary for nocturnality in LD. More specifically, Or47b ORNs are necessary for the manifestation of this phenotype, and it is likely that Or65a and Or88a ORNs are also involved. Finally, consistent with MSDR in DD (Fujii et al., 2007), males play a key role in driving increased MF nighttime activity. However, females might also be involved. It is thus evident that the male clock mechanism plays a crucial role in integrating and responding to social signals emanating from the female and detected by olfaction in DD. In LD, however, the role of the male circadian clock in coordinating the social response following olfactory detection is not confidently established or understood. Further research is thus necessary in this regard.

2.4 LONG-TERM RESPONSES TO THE SOCIAL ENVIRONMENT

2.4.a PAIRWISE MM INFLUENCE ON LONG-TERM ACTIVITY RHYTHMS

One paradigm in which the influence of social interactions on activity has been studied involves testing whether cyclic presence and absence (PA) of conspecifics affects long-term (as opposed to immediate) host locomotor activity in a pair setting. When males are entrained in LD, switched to DD, then subjected 12 hours a day to a male companion from a different LD regime, an influence on subsequent activity in DD is observed (Lone et al., 2011). More specifically, a significant change in average phase occurs, as well as a decrease in collective phase coherence.

The extent of this influence depends on the visitor’s LD regime relative to that of the host. Notably, loss of function per visitors from an LD regime do not induce a change in phase coherence, although a change in average phase persists (Lone et al., 2011). This indicates that per and thus the visitor’s circadian system is involved in modulating the hosts’ phase as a consequence of cyclic visitations. Whether the visitor’s entrainment plays a direct role by simply modulating its activity state, which
somehow influences the host, or whether another downstream behaviour is clock-controlled and inflicts a social effect on the host remains to be determined.

Given that a host male’s clock status plays an integral role in processing the presence of a visiting female and responding accordingly in DD and possibly in LD, the question becomes whether a male host’s circadian clock plays a similar role in modulating long-term activity changes in response to a visiting male. This question has been only tentatively addressed. The long-term change in activity as a result of social experience is only observed when visitations are conducted during portions of the host’s subjective day and night. When visitations take place during the host’s subjective day, no influence on phase is detected. This indicates that this long-term social influence is not only dependent on the visitor’s entrainment, but also on the time at which the social interaction occurs relative to the host. That host entrainment determines whether a host responds to a visitor indicates that it is possible that the host’s receptivity is clock-dependent. For instance, clock-controlled olfactory rhythms might be involved in conspecific detection. Another experiment hints to the necessary role of a male host’s circadian clock in responding to a male conspecific. When exposed to cyclical visits by WT males, phase coherence of flies with labile rhythmicity (Pdf-gal4/UAS-shibirets) remains unaltered, whereas a significant phase shift occurs (Lone et al., 2011). Before interpreting these findings, it is important to clarify that visitations in this assay occur during the hosts’ subjective day as opposed to visits that last for a portion of the subjective day and night. Also of note is that the control chosen by the authors underwent visitations during the latter treatment which is not the appropriate choice. The more relevant control showed no influence on phase under these conditions. When this relevant control is considered, labile flies display a phase shift indicative of the host’s clock response to male companionship. This suggests that the host’s circadian clock is also involved in the manifestation of this long-term social influence on activity.

Collectively, these experiments indicate that MM pairings affect long-term locomotor activity. An examination of the role of the host’s circadian system offers preliminary evidence that it is involved in this long-term social response. On the visitor’s end, an intact visitor molecular oscillator is necessary for a long-lasting social response to occur. Whether the visitor’s entrainment plays a direct role by simply modulating its activity state which influences the host, or whether another downstream behaviour is clock-controlled inflicting a social effect on the host remains to be determined. Although these findings do not provide sufficient evidence for a solid model, they highlight an interesting concept. The circadian clocks of both visitor and host are required for this social response. One
possibility is that synchrony is mediated by the circadian timing systems of both individual (i.e., both clocks ‘communicate’ to generate a coordinated long-lasting effect). This seems likely given that the entrainment of the visitor does not completely override the host’s.

2.4.b INFLUENCE OF HETEROSEXUAL GROUPINGS ON LONG-TERM LOCOMOTOR ACTIVITY

In a group setting, MF interactions referred to hereon after as socio-sexual interactions (SSI), also influence activity rhythms in LD (Lone and Sharma, 2011a). Activity in this case is measured after the social experience as opposed to during. Similar to MSDR (Fujii and Amrein, 2010; Fujii et al., 2007), males undergo a reduction in evening activity post-group SSI and a lengthening of circadian period despite the strong Zeitgeber role of light. Females, on the other hand, undergo an overall decrease in activity. A minimum of four days of SSI is necessary for such effects to occur, beyond which further exposure does not enhance group SSI after effects. As a result, in contrast to MSDR, which is not entrained and requires the constant presence of a female (Fujii et al., 2007), group SSI have a long-lasting effect. It is premature to attribute such differences to group size because MSDR was measured in the OR strain whereas post-group interaction activity was studied in CS. Furthermore, although locomotor activity in an MF pair was tested in several strains, the existence of a long-lasting effect in these strains was never verified to warrant such a conclusion (Lone and Sharma, 2012).

Males from loss of function per and cry MF groups in LD do not display SSI aftereffects in LD (Lone and Sharma, 2011a). Furthermore, males from WT MF groups in constant light conditions (LL) do not display SSI aftereffects in LD either. This implies that an intact clock in males is necessary for the establishment of SSI aftereffects. As pairwise MSDR is abolished in clock mutant males MSDR (Fujii and Amrein, 2010; Fujii et al., 2007), it is likely that group SSI in clock mutants interacting in LD and WT individuals interacting in LL undergo arrhythmic interactions. Therefore, the male circadian clock might be necessary for rhythmic group SSI, and this rhythmicity might in turn be necessary for post-SSI activity changes. However, the rhythmicity of group SSI and its necessity for long-lasting aftereffects remains to be tested directly.

Notably, females from loss of function per and cry mutant MF groups in LD and females from WT MF groups in LL show normal SSI aftereffects in LD. This implies that female clocks are not required for such a social response and that the latter might be driven by non-circadian factors. The authors of this finding stipulate that such changes might be driven by factors such as accessory gland proteins that
are deposited into the female reproductive tract during mating, which could potentially tune down their physiology and behaviour (Gillott, 2003; Kubli, 2003; Yapici et al., 2008).

One reservation is warranted with regards to these findings and interpretations. Both male and female clock mutants were placed together to investigate the role of clocks in the mediation of group SSI aftereffects on both genders. In the case of females, this is not problematic, as the interpretation of these results can be extended to say that both male and female clocks are not required for female post-group SSI effects. In the case of males, however, interpretation of the findings becomes problematic because two variables were altered at the same time. In other words, if males cease to respond to previous group SSI when male and female clocks have been manipulated, the role of female clocks cannot be deemed irrelevant. Having said that, as MF pair activity is mostly male driven (Fujii et al., 2007; Lone and Sharma, 2012) and females from MF clock mutant groups continue to respond to the social treatment, it is indeed likely that group SSI aftereffects are also driven by the male clock. However, this cannot be confidently concluded until clock mutant males are tested with WT females.

As the LN-s play a significant role in MSDR (Fujii and Amrein, 2010), they constitute a strong candidate in mediating individuals’ social response to group SSI. Surprisingly, males with electrically silenced LN-s continue to respond to group SSI by reducing their evening activity peak (Lone and Sharma, 2011a). It is noteworthy that Pdf-gal4 was used to silence the LN-s in these experiments despite previous findings doubting the strength of this driver. It is thus necessary to test the supposedly more active driver Mz520-gal4. In LN-silenced females, nighttime activity is unaffected, whereas daytime activity is reduced (Lone and Sharma, 2011a). This is surprising given that female clocks were deemed irrelevant to this social response. It is possible that a non-clock-related function in the LN-s has a role in reducing daytime activity in SSI females. This effect supports the potency of this previously doubted driver. Needless to say, the use of Mz520-gal4 in addition to other available tools is necessary to resolve accumulating doubts and to increase confidence in these conclusions.

Blocking olfactory responses using Or83b mutants or individuals with ablated Or83b ORN using the apoptotic gene reaper, abolishes males’ long-term social response to group SSI (Lone and Sharma, 2011a). Females with a manipulated olfactory system, on the other hand, do not alter long-term nighttime activity but still reduce their daytime activity. Again, the simultaneous use of males and females with compromised olfactory systems makes the interpretation of these findings tricky. We can cautiously conclude that the male olfactory system is necessary for female recognition in order to
trigger long-term activity changes. However, this needs to be confirmed by employing olfactory mutant males with WT females. The same applies to females. It can be generally stated, though, that olfaction is necessary for mediating social influences on long-term activity.

Together, the findings indicate that similar to MSDR, group SSI also influence activity rhythms of males and females in LD. In contrast to MSDR, which is not entrained and requires the constant presence of a female, group SSI have a long-lasting impact. It is premature to attribute such differences to group size. Among other reasons, MSDR and post-group interaction activity were examined in different strains.

An intact clock in males is necessary for the establishment of SSI after effects. It is possible that an intact circadian clock is necessary for rhythmic group interaction, as is the case with MSDR, and this rhythmicity might in turn be necessary for post-SSI activity changes. Female clocks, on the other hand, might not be required for such a social response. It is possible that this effect is driven by non-circadian factors. However, the design of the assay is inappropriate to generate solid evidence in that regard. It should be noted that the role of clocks in the mediation of this effect was dissected further in this assay. For example, males with electrically silenced LN_v continue to respond to group SSI. However, Pdf_gal4 was used to silence the LN_v and the use of Mz520-gal4 in addition to other available tools is necessary to increase confidence in these conclusions.

Lastly, it can be generally stated that olfaction is necessary for mediating the influence of SSI on long-term activity. Whether male and/or female olfaction is needed, remains to be confirmed. This is because the design of the assay employed cannot resolve whether one or both are required.

2.4.c INFLUENCE OF ISOLATION AND GROUP COMPOSITION ON LONG-TERM LOCOMOTOR ACTIVITY

Long-term mean circadian phase (defined as average peak time of locomotor activity) of group-reared WT males does not differ from WT individuals reared in isolation (Levine et al., 2002a). Phase coherence on the other hand, which reflects within treatment phase similarity, is greater in group-reared individuals compared to isolates (Levine et al., 2002a; Lone and Sharma, 2011b). An increase in phase coherence also occurs when individuals with variable entrainments are co-housed and subsequently compared to their isolated equivalents (Lone and Sharma, 2011b). This indicates that the long-term circadian phase of group-housed individuals is fine-tuned and synchronized as a result of prior cohabitation. Given that locomotor activity is clock-controlled, it follows that clocks of group-
housed individuals are likely to achieve higher synchrony than those raised in isolation through inter-individual clock interactions. The role of clocks in this improved synchrony is further validated by housing arrhythmic per\(^0\) mutants with WT males. Long-term locomotor activity of WT hosts raised with per\(^0\) visitors (heterogeneous group) is significantly altered in comparison to males raised in a WT homogeneous group. Not only does the presence of per\(^0\) visitors decrease phase coherence between individuals, but mean phase is also significantly altered (Levine et al., 2002a). This strongly suggests that changes in activity as a result of group housing is mediated through clock-related mechanisms within the visitors. On the other hand, a decrease in overall group phase coherence occurs when individuals with variable per alleles, such as per\(^L\), are co-housed (per\(^ S\)+per\(^L\), per\(^ S\)+WT or per\(^ L\)+WT) and compared to their homogeneous group equivalents (Lone and Sharma, 2011b).

Other social manipulations also inflict a change on phase. Visitors from an earlier time zone, as well as per\(^S\) visitors inflict a phase change on the long-term locomotor activity of per\(^L\) and late hosts respectively (Levine et al., 2002a). Notably, per\(^L\) and late visitors do not influence phase of per\(^S\) and early hosts respectively. This points to an interesting directionality in these social interactions. It has been suggested that such individuals with delayed activity profiles might be more sensitive to the presence of faster cohabitants than the other way round (Lone and Sharma, 2011b). Regardless, it is clear that clocks within hosts are involved in the manifestation of activity changes in response to social interactions. The influence of the social environment, such as presence of per\(^0\) visitors on hosts’ clocks, is further reinforced by changes in host brain gene expression of several clock genes—per, tim, and Clk (Krupp et al., 2008). Consequently, just in the MM pair setting, it seems that circadian clocks of both visitors and hosts are required for this social response with the possibility of both clocks ‘communicating’ to generate a coordinated long-lasting effect. Interestingly, these long-term social influences are dependent on the ratio of hosts and visitors, adding another characteristic dimension of the social environment that impacts long-term activity (Levine et al., 2002a).

The sensory mechanisms underlying this social effect on circadian function have been investigated. Given that visitors interact with hosts in DD conditions, it is unlikely that visual stimuli play a role (Levine et al., 2002a). Olfaction, on the other hand, does play a role. When humidified air is pumped through a vial containing WT individuals and received by isolates, locomotor activity of these isolates is more synchronized in comparison to isolates receiving air pumped from an empty vial (Levine et al., 2002a). Furthermore, long-term locomotor activity of olfactory mutants is not influenced by these social treatments (Levine et al., 2002a; Lone and Sharma, 2011b). Such olfactory signals have been previously
highlighted as socially sensitive (Kent et al., 2008), and it has been suggested that they mediate social information, such as species, gender, and mating status (Billeter et al., 2009). Within males, these include compounds such as (Z)-7-tricosene (7-T), also known as C23:1(7) (7-T), as well as cVA. Not only is olfaction required for the mediation of this social influence, but the temporal regulation of olfactory input is also necessary. This is the case because per7.2 hosts with per expression restricted to certain clock neurons and thus devoid of temporal olfactory regulation (Frisch et al., 1994) fail to respond to the presence of per2 visitors (Levine et al., 2002a). This indicates that temporal regulation of sensory input is essential for mediating this social response. It is possible that auditory and tactile cues also play a role in the communication of these social conditions; however, this remains untested.

To sum up this section, the long-term circadian phase of group-housed males is fine-tuned and synchronized as a result of prior cohabitation. Consequently, just as in the MM pair setting, it seems that circadian clocks of both visitors and hosts are required for this social response with the possibility of both clocks ‘communicating’ to generate a coordinated long-lasting effect within a group of males. These long-term social influences are dependent on the ratio of hosts and visitors, adding another characteristic dimension of the social environment that impacts long-term activity. It seems that not only is olfaction required for the mediation of this social influence, but the temporal regulation of olfactory input is also necessary.

2.5 CONCLUSIONS REGARDING SOCIAL EFFECTS ON D. MELANOGASTER LOCOMOTOR ACTIVITY

Several studies have focused on understanding the role of clocks in mediating immediate and long-term effects of social interactions on activity. Since activity encompasses other behaviours, these findings might prove informative in future investigations regarding the mechanisms with which an individual adjusts other behaviour(s) in response to the social surroundings.

Locomotor activity of D. melanogaster individuals is altered in the presence of a conspecific. The immediate consequences of MM and FF pairings on locomotor activity have been minimally investigated; however, it is known that they significantly differ from MF couples. This implies that the social environment modulates an individual’s activity in a gender-specific manner.

MF pairings on the other hand have been thoroughly considered. Based on the available reliable studies investigating MF pairs, it can be confidently concluded that the presence of the female is detected by the male through olfaction, triggering courtship behaviour. The change in male activity
due to female-induced courtship is not additively overlaid on male SLR, does not operate within the boundaries of SLR, and does not override SLR irrespective of male clock status. Instead, the male circadian clock plays a key role. The female social signal is integrated within the male’s internal clock in the LN, s and at least some DN1s. The role of the time-keeping system in modulating courtship and mating behaviour is not surprising. Differences in timing of mating behaviour of *D. melanogaster* and *D. pseudoobscura* have been documented and have been shown to be dependent on the *per* gene (Tauber et al., 2003).

As observed in DD, MF couples display increased nighttime activity levels in LD, and this increase is also associated with courtship. In contrast with DD settings, however, intact male circadian molecular oscillations are not required for the establishment of this behaviour in LD. Instead, clock coherence, regardless of rhythmicity, as well as non-clock-related functions in clock neurons might be necessary for this nocturnality. Thus, how a male’s circadian clock coordinates this social response in LD remains unknown. It is worth noting that females might also play a role in this social response. Consistent with DD settings, olfaction is necessary for nocturnality in LD. Specifically, *Or47b* ORNs are necessary for the manifestation of this phenotype, and *Or65a* and *Or88a* ORNs are also involved.

MM pairings affect long-term locomotor activity. The role of the host’s circadian system has been examined providing preliminary evidence that it is involved in this long-term social response. It is possible that the host’s receptivity is clock-dependent because the timing of the social experience determines whether a long-term effect is observed or not. On the visitor’s end, an intact molecular oscillator is also necessary for a long-lasting social response. Whether the visitor’s entrainment plays a direct role by modulating visitor activity which in turn influences the host or whether another downstream behaviour is clock-controlled inflicting a social effect on the host remains to be determined. Although these findings do not provide sufficient evidence for a solid model, they highlight an interesting notion where the circadian clocks of both visitor and host ‘communicate’ to generate a coordinated long-lasting effect. This is likely given that the entrainment of the visitor does not completely override the host’s.

Long-lasting social influence on activity patterns has been investigated in heterosexual and same-sex group contexts. In contrast to MSDR, which is not entrained and requires the constant presence of a female, group SSI have a long-lasting effect on activity even in LD. It is premature to attribute such differences to group size. Notably, an intact clock in males is necessary for the establishment of SSI
aftereffects. It is possible that an intact circadian clock is necessary for rhythmic group interactions, as is the case with MSDR, and this rhythmicity might in turn be necessary for post-SSI activity changes. However, this remains to be tested directly. Males with electrically silenced LNvs continue to respond to group SSI. Unfortunately, only Pdf-gal4 was used to silence the LNvs and the use of Mz520-gal4 in addition to other available tools is necessary to increase confidence in these conclusions.

Female clocks might not be required for such a social response. It is possible that this effect is driven by non-circadian factors. However, the design of the assay with which this question was examined was inappropriate to generate solid evidence in that regard. It can be stated, however, that olfaction is necessary for mediating the influence of SSI on long-term activity. Whether male and/or female olfaction is needed, however, remains to be confirmed. This is because the design of the assay employed cannot resolve whether one or both are required.

Same-sex group social interactions are influential on long-term activity patterns. More specifically, the long-term circadian phase of group-housed males is fine-tuned and synchronized as a result of prior cohabitation. In the MM pair setting, it seems that circadian clocks of both visitors and hosts are required for this social response with the possibility of both clocks ‘communicating’ to generate a coordinated long-lasting effect. Notably, these long-term social influences are dependent on the ratio of hosts and visitors, adding another characteristic dimension of the social environment that impacts long-term activity. Finally, not only is olfaction required for the mediation of this social influence, but the temporal regulation of olfaction is also necessary.

To sum up, a few themes emerge from the collective consideration of these studies. First, a variety of social treatments are influential on locomotor activity patterns. These social contexts include heterosexual and same-sex pairs and groups. Some social effects are immediate whereas others are long lasting. Most of the social treatments considered seem to be mediated by olfaction. Clocks appear to be integral in mediating most social influences on activity. This is not surprising, as locomotor activity is a well-known clock-influenced behaviour. Furthermore, the mechanism involved in detecting conspecifics in the social environment, as well as the mechanism by which their presence is incorporated into an individual state differs between LD and DD. It is also plausible that circadian clocks act as a hub for the integration of social signals and the appropriate modulation of downstream behaviours, be it courtship or other unidentified interactions. Finally, although a fair number of studies have been considered on this topic, this line of questioning is still in its infancy. Progress has been
hindered by the inconsistency in assays, strains, and conditions used to build on pre-established findings. Although the literature reviewed here has provided a preliminary and widespread backbone for the genetic and neural substrates involved in the manifestation of social influence under various conditions, investigations can be made more efficient. It is important to take previous findings into account, examine outstanding conclusions, and conduct the necessary investigations to begin solidifying understanding in these areas. For that to occur, consistency in the assays employed is essential for findings to interlock confidently. A review of the studies suggest that well-designed paradigms and appropriate controls would benefit the field. This line of questioning has a rich variety of tools available and their employment will permit the generation of knowledge despite the downsides associated with certain techniques.
2.6 Chapter 2 Tables

Table 2-1 | Summary of studies investigating the influence of the social environment on locomotor activity.

<table>
<thead>
<tr>
<th>Paper</th>
<th>Pair</th>
<th>Group</th>
<th>During social experience</th>
<th>After social experience</th>
<th>Measure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fujii et al., 2007</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
<td>Velocity, Close-proximity</td>
</tr>
<tr>
<td>Fujii and Amrein, 2010</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
<td>MSDR, CRI</td>
</tr>
<tr>
<td>Lone and Sharma, 2010</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
<td>Close-proximity</td>
</tr>
<tr>
<td>Lone and Sharma, 2012</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
<td>Locomotor activity</td>
</tr>
<tr>
<td>Hanafusa et al., 2013</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
<td>Locomotor activity (MF)</td>
</tr>
<tr>
<td>Lone and Sharma, 2011b</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
<td>Locomotor activity (MM)</td>
</tr>
<tr>
<td>Lone et al., 2011</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
<td>SLR</td>
</tr>
<tr>
<td>Levine et al., 2002a</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
<td>SLR</td>
</tr>
<tr>
<td>Lone and Sharma, 2011a</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
<td>SLR</td>
</tr>
<tr>
<td>Lone and Sharma, 2011b</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
<td>SLR</td>
</tr>
</tbody>
</table>

This table summarizes the social contexts investigated (pair or group) and whether the social impact examined occurs immediately or is long-lasting. The measures used in each of the studies have also been included and are defined in the List of Acronyms on page xiii, and within the text of Chapter 2. Briefly, MSDR stands for male sex drive rhythm. CRI stands for courtship rest index, calculated by dividing the mean of courtship during the whole day by that of a single male and female cohabitation. PDR stands for single male locomotor rhythm. MF represents a male-female social context, whereas MM indicates a male-male social context.

Table 2-2 | Summary of treatments investigating the role of different clock neurons in mediating the social influence of male-female cohabitation.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>slN.s</th>
<th>LN.s</th>
<th>S(LN.s)</th>
<th>DN1</th>
<th>DN2</th>
<th>DN3</th>
<th>Effect on MF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pdf-gal4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Amplitude slightly dampened*</td>
</tr>
<tr>
<td>cry-gal4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Dusk dip and night activity &amp; ↓ rhythmicity*</td>
</tr>
<tr>
<td>cry-gal4, Pdf-gal80</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Arrhythmic</td>
</tr>
<tr>
<td>npf-gal4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lone and Sharma, 2010</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Rows 1-4</td>
</tr>
<tr>
<td>tim-gal4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Arrhythmic</td>
</tr>
<tr>
<td>Pdf-gal4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Slight Amplitude ↓↓ night activity &amp; ↓↓ rhythmicity by 3rd day</td>
</tr>
<tr>
<td>cry-gal4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Amplitude slightly dampened*</td>
</tr>
<tr>
<td>cry-gal4, Pdf-gal80</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Rhythm altered to show 12 hr. period*</td>
</tr>
<tr>
<td>npf-gal4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lone and Sharma, 2010</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Rows 5-9</td>
</tr>
<tr>
<td>slov-gal4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>↓↓↓ CRI</td>
</tr>
<tr>
<td>cry-gal4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>↓↓ CRI</td>
</tr>
<tr>
<td>tim-gal4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>↓↓ CRI</td>
</tr>
<tr>
<td>E70-gal4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>↓↓ CRI</td>
</tr>
<tr>
<td>Pdf-gal4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ms200-gal4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Strong MSDR disruption</td>
</tr>
<tr>
<td>fru-gal4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>↓↓ CRI</td>
</tr>
<tr>
<td>fru-gal4, Pdf-gal80</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Strong MSDR disruption</td>
</tr>
<tr>
<td>fru-gal4, cry-gal80</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>No effect</td>
</tr>
<tr>
<td>C319-gal4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>↓↓ CRI</td>
</tr>
<tr>
<td>Pdf4.4gal4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>↓↓ CRI</td>
</tr>
<tr>
<td>Lone and Sharma, 2010</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Rows 10-20</td>
</tr>
<tr>
<td>Pdf50</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>↓ CRT</td>
</tr>
<tr>
<td>per6, slov-gal4, Pdf-gal80</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Strong MSDR disruption</td>
</tr>
<tr>
<td>per6, Ms200-gal4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Altered MSDR</td>
</tr>
<tr>
<td>per6, cry-gal4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Altered MSDR</td>
</tr>
<tr>
<td>per6, fru-gal4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Altered MSDR</td>
</tr>
<tr>
<td>per6, C319-gal4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>↓↓ MSDR Occurrence</td>
</tr>
<tr>
<td>per6, C4.4F gal4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>↓↓ MSDR Occurrence</td>
</tr>
<tr>
<td>per6, C319 &amp; Ms200-gal4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lone and Sharma, 2010</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Rows 21-28</td>
</tr>
</tbody>
</table>

* Refers to descriptive effects described by authors yet lacking statistical confirmation. Rows with red boxes attempt to conclusively mark neuronal clusters that seem to play a role.

- Indicates fully manipulate cluster of cells
- Indicates a subset of cells is affected (lighter colored version implies less cells are affected
- All but one or 2 cells affected
- One or 2 cells only are affected
- Implies that it can be concluded that cells are involved in process
- Subset of cells is involved in process
- Role of cells is inconclusive
- Either or both clusters are involved
Chapter 3  SPECIALIZED CELLS TAG SEXUAL AND SPECIES IDENTITY IN DROSOPHILA MELANOGASTER


This chapter was originally published in 2009 in Nature. I am the second author on that paper. Together with Dr. Billeter, the first author, I collected and analyzed all data, and worked on the revised manuscript. This paper highlights the importance of cuticular hydrocarbons in social signaling and recognition, which constitute essential precursors to social modulation of behaviour. It also establishes methods and details that are used in subsequent chapters. Permission to reproduce this manuscript is included in APPENDIX A.V.2.

3.1 INTRODUCTION

Social interactions depend on individuals recognizing each other, and in this context many organisms use chemical signals to indicate species and sex (Wyatt, 2003). Cuticular hydrocarbon signals are used by insects, including Drosophila melanogaster, to distinguish conspecific individuals from others (Ferveur, 2005; Jallon, 1984; Wyatt, 2003). These chemicals also contribute to intraspecific courtship and mating interactions (Ferveur, 2005; Jallon, 1984; Wyatt, 2003). However, the possibility that sex and species identification are linked by common chemical signaling mechanisms has not been formally tested. Here we provide direct evidence that a single compound is used to communicate female identity among D. melanogaster, and to define a reproductive isolation barrier between D. melanogaster and sibling species. A transgenic manipulation eliminated cuticular hydrocarbons by ablating the oenocytes, specialized cells required for the expression of these chemical signals. The resulting oenocyte-less (oe-) females elicited the normal repertoire of courtship behaviours from males, but were actually preferred over WT females by courting males. In addition, WT males attempted to copulate with oe- males. Thus, flies lacking hydrocarbons are a sexual hyperstimulus. Treatment of virgin females with the aversive male pheromone cVA significantly delayed mating of oe- females compared to WT females. This difference was eliminated when oe- females were treated with a blend of cVA and the female aphrodisiac (7Z, 11Z)-heptacosadiene (7,11-HD), showing that female aphrodisiac compounds can attenuate the effects of male aversive pheromones. 7,11-HD also was shown to have a crucial role in heterospecific encounters. Specifically, the species barrier was lost because males of other Drosophila species courted oe- D. melanogaster females, and D. simulans
males consistently mated with them. Treatment of oe-females with 7,11-HD restored the species barrier, showing that a single compound can confer species identity. These results identify a common mechanism for sexual and species recognition regulated by cuticular hydrocarbons.

3.2 RESULTS AND DISCUSSION

*D. melanogaster* produces hydrocarbons of various chain lengths, including unbranched alkanes, methyl-branched alkanes, alkenes and derivatives thereof. The alkenes are expressed sex-specifically, and have been associated with both sex and species discrimination (Coyne et al., 1994; Ferveur, 2005; Jallon, 1984; Savarit et al., 1999). Compared to females, males express high levels of the monoalkene (Z)-7-tricosene (7-T), which has been reported to increase females’ receptivity to mating attempts (Grillet et al., 2006). Moreover, 7-T is repulsive to other males and may prevent male–male interactions (Lacaille et al., 2007). In contrast, females produce sex-specific dienes such as 7,11-HD and (7Z, 11Z)-nonacosadiene (7,11-ND), which act as aphrodisiac pheromones for *D. melanogaster* males (Antony et al., 1985; Jallon, 1984). Hydrocarbons are strongly associated with sexual recognition, because WT males court males that have been genetically modified to express female hydrocarbons, indicating that the mutants are perceived as females (Ferveur et al., 1997).

There are still large gaps in our knowledge of the functions of individual hydrocarbons and the tissues where these compounds are synthesized. As in other insects (Fan et al., 2003), specialized cells called oenocytes, located on the inner surface of the abdominal cuticle, are thought to be the site of hydrocarbon biosynthesis in *D. melanogaster* (Ferveur, 2005). Consistent with this hypothesis, desaturase 1 (*desat1*), which encodes an enzyme involved in hydrocarbon synthesis (Marcillac et al., 2005), is expressed in *Drosophila* oenocytes (Krupp et al., 2008) (Figure 3.1 A). Previous studies have demonstrated that genetic feminization of these cells results in production of female hydrocarbons by male flies (Ferveur et al., 1997); however, these and other manipulations have been confounded by the concurrent feminization of cells in many other sexually dimorphic tissues, including the central nervous system (Ferveur et al., 1997; Savarit et al., 1999). To test the hypothesis that these cells are required for production of chemical signals used in sexual and species recognition, we used the Gal4-UAS system (Venken and Bellen, 2005) to target transgene expression specifically to the adult oenocytes. We generated an oenocyte Gal4 driver (Figure 3.1 B) derived from the regulatory sequence of one of the *desat1* promoters (Marcillac et al., 2005) that is expressed specifically in oenocytes of adult females (Figure 3.1 A-C). The driver is also expressed in the larval oenocytes and in the reproductive
organs of adult males (Figure 3.1 A and C and Online Supplementary Figure 1). We used this driver to ablate adult oenocytes by inducing expression of the pro-apoptotic gene head involution defective (hid; also called Wrinkled) (Zhou et al., 1997). This approach initially caused lethality in larvae, probably due to the destruction of the larval oenocytes (Gutierrez et al., 2007). To circumvent this problem we blocked the driver’s action during development using the Tubulin-Gal80ts transgene (McGuire et al., 2003). Using this method, we generated adult flies without oenocytes (oe-) (Figure 3.1 D and E). Analysis of whole-body hydrocarbon extracts confirmed that both oe- males and females were essentially devoid of these compounds (Figure 3.1 F and G and Online Supplementary Tables 1 and 2 for quantification), showing that the oenocytes are necessary for hydrocarbon display in D. melanogaster. The male pheromone cVA was unaffected in oe- males (Figure 3.1 F and G) because this compound is synthesized in the ejaculatory bulb (Butterworth, 1969). The oe- transgenic strain therefore provided a ‘blank slate’ for evaluating the role of hydrocarbons in intra- and interspecific communication.

We assayed sexual behaviour of oe- flies to test hydrocarbon function during reproduction. Despite the association of hydrocarbon signals and Drosophila courtship, absence of these signals did not alter courtship behaviours per se. The oe- males displayed normal courtship behaviour towards WT females, but slightly less intense than control males (Figure 3.2 A and Online Supplementary Table 3). However, WT females were less receptive to oe- males than control males, with oe- males taking almost four times as long to achieve mating (Figure 3.2 A). Thus, hydrocarbons of males do not seem to affect their own courtship behaviour, but rather, influence the receptivity of females to their mating attempts. However, we cannot exclude the influence of non-oenocyte cells within the male reproductive organs that may have been affected by the ablation. Notably, oe- males elicited courtship and copulation attempts from both WT males and other oe- males, indicating that oe- males were perceived as females, even though all other male characteristics were present (Figure 3.2 B and Online Supplementary Table 4). The vigorous courtship of oe- males by each other resulted in unnatural behaviours such as engaging one another by rotating in a head-to-head orientation, and males attempting copulation with each other’s heads (Figure 3.2 B, Online Supplementary Table 4 and Supplementary Movies 1 and 2). These behaviours were suppressed by treatment of oe- males with synthetic 7-T (Figure 3.2 B and Online Supplementary Table 4), confirming the function of 7-T in inhibiting male–male interactions (Lacaille et al., 2007).
WT males exhibited normal courtship behaviour towards oe- females, apparently undeterred by the lack of female hydrocarbons (Figure 3.2 C). However, mating latency was significantly shorter (Figure 3.2 C), and when given a choice between an oe- and a control female, WT males preferred oe- females (Figure 3.2 D). Together, these data indicate that females lacking hydrocarbons are more attractive than those with a normal hydrocarbon profile. This suggests that female hydrocarbons normally act to slow down male mating attempts, facilitating assessment of a potential partner’s species and fitness. Thus, any oe- fly, irrespective of its development as female or male, seems to sexually hyper stimulate males. We hypothesize that hydrocarbons normally act to superimpose sexual identity on an otherwise attractive fly substrate.

The results described above suggested that female attractiveness depends on a balance between attraction/stimulation and repulsion/ deterrence. We investigated this by treating females with the aphrodisiac compound 7,11-HD, and with cVA, which males transfer to females via the ejaculate (Jallon, 1984; Mane et al., 1983) to deter further mating attempts by other males (Kurtovic et al., 2007; Mane et al., 1983). Whereas cVA decreases the probability that females will remate, wild-caught females produce offspring from multiple sires, indicating that polyandry is common (Imhof et al., 1998) and that the effect of cVA is not absolute. We treated oe- flies with doses of these compounds approximating WT levels (see Methods). The mating latency of WT males with oe- females treated with 7,11-HD was not different from that with untreated oe- females ($p > 0.7$), indicating that 7,11-HD alone does not affect attractiveness of oe- females (Figure 3.2 E). As expected, treating WT females with increasing doses of cVA delayed mating accordingly (Figure 3.2 F), and the effect was even more pronounced with oe- females treated with the same doses of cVA (Figure 3.2 F). This effect was not due to differences in the rates of release of cVA from the control and oe- flies, as shown by the profiles of cumulative loss of cVA over time for the two genotypes (Online Supplementary Figure 2). Instead, the exaggerated effect of cVA on oe- females is consistent with our hypothesis that the aversive effects of this compound are normally moderated by the presence of other hydrocarbons. Indeed, when cVA and 7,11-HD were applied together, the mating latencies of oe- and WT females were indistinguishable (Figure 3.2 G). Apparently, 7,11-HD mitigated the deterrent effects of cVA. The data suggest that a male’s perception of a female’s availability is normally regulated by a mixture of attractive and aversive signals (Figure 3.2 F and G). From an evolutionary perspective, the combined effect of a female attractant with a male deterrent may illustrate an instance of post-copulatory sexual conflict (Hosken et al., 2009) in
which the attractant solicits additional mates despite the first male’s effort to render a female unattractive by marking her with cVA.

In addition to mediating conspecific reproductive interactions, the hydrocarbons of female *D. melanogaster* have an important role in reproductive isolation between species (Coyne et al., 1994; Ferveur, 2005; Savarit et al., 1999). For example, within the nine species of the *melanogaster* subgroup, only *D. melanogaster*, *D. sechellia* and *D. erecta* produce female-specific dienes (Ferveur, 2005; Jallon and David, 1987). Females in the rest of the subgroup express the same hydrocarbons as males (Ferveur, 2005; Jallon and David, 1987). Males of species with non-sexually dimorphic hydrocarbons generally do not court females from dimorphic species, indicating that the dienes might act as reproductive isolation barriers between these species groups (Cobb and Jallon, 1990; Coyne et al., 1994). Furthermore, males from dimorphic species do not vigorously court females from non-dimorphic species (Cobb and Jallon, 1990; Coyne et al., 1994; Jallon, 1984). In contrast, males of all species in the *melanogaster* subgroup have similar hydrocarbons, including abundant 7-T (Jallon and David, 1987). Finally, *D. melanogaster* females lacking hydrocarbons are courted by at least two sibling species, *D. simulans* and *D. mauritiana* (Savarit et al., 1999). We tested the behaviour of males from other species in the *melanogaster* subgroup towards oe- females, to assess the contribution of oenocytes and hydrocarbons to reproductive isolating mechanisms. We chose *D. simulans* and *D. yakuba* as test species because they represent species in which the females lack dienes. We included *D. erecta* because it differs from *D. melanogaster* in the pattern of dienes expressed (Jallon and David, 1987) (Figure 3.3 A).

Males of all three species courted oe- *D. melanogaster* females, but exhibited limited or no courtship towards control *D. melanogaster* females (Figure 3.3 B and Online Supplementary Table 5). This indicates that oenocytes and their hydrocarbon products are major components of the reproductive isolation barrier, ensuring that courtship and mating attempts are only initiated between conspecifics. It has been proposed that 7,11-HD functions to create this barrier in *D. melanogaster* (Coyne et al., 1994; Savarit et al., 1999). To test this directly, oe- *D. melanogaster* females and WT females from the different species were treated with synthetic 7,11-HD. Treatment suppressed courtship by males of all three species (Figure 3.3 B and Online Supplementary Table 5), demonstrating that 7,11-HD alone is sufficient to create a species barrier. Interestingly, *D. erecta* males were blocked by 7,11-HD (Figure 3.3 B and Online Supplementary Table 5), despite the fact that hydrocarbons of *D. erecta* females include other dienes in common with those of *D. melanogaster* (Figure 3.3 A). Furthermore, *D. melanogaster* males actively
courted *D. erecta* females (Figure 3.3 C), possibly because the diene 7,11-ND is also expressed by *D. melanogaster* females (Cobb and Jallon, 1990; Jallon and David, 1987) (Figure 3.3 A). *D. simulans* and *D. yakuba* females treated with 7,11-HD elicited strong courtship from *D. melanogaster* males (Figure 3.3 C and Online Supplementary Table 5). These results demonstrate the multifunctional role of 7,11-HD as an attractant and/or stimulant for some species and as a deterrent for others.

Despite attempting copulation (Online Supplementary Table 5), *D. erecta* males never mated with oe-females, suggesting that signals other than hydrocarbons are required to induce receptivity in these females (Cowling and Burnet, 1981). However, within a 24-h period, nearly all oe-*D. melanogaster* females mated with *D. simulans* males, whereas no control *D. melanogaster* females mated with these males (Figure 3.4 A). Treatment of oe- females with 7,11-HD completely blocked interspecific mating with *D. simulans* males, even at a dose five times lower than the amount found in females of our WT *D. melanogaster* strain (Figure 3.4 B). Similar treatment of *D. simulans* females with 7,11-HD only delayed mating by *D. simulans* males (Figure 3.4 B). We hypothesized that 7-T counters the effect of 7,11-HD in *D. simulans* females. This is because 7-T functions as an aphrodisiac for *D. simulans* males and is expressed in higher quantities in *D. simulans* females than in *D. melanogaster* females (Jallon, 1984). We assayed *D. simulans* males with oe- females treated with either 7-T alone, or in combination with 7,11-HD. Synthetic 7-T alone induced a slight decrease in mating latency, indicating that 7-T is an attractant for *D. simulans* males (Figure 3.4 C). However, the striking effect of 7-T was to reduce the effect of 7,11-HD in a dose-dependent manner (Figure 3.4 C). These data parallel the balancing effect of 7,11-HD on cVA for *D. melanogaster* males. Thus, we have demonstrated that female hydrocarbons orchestrate mating both within and between the species. Whereas a single compound such as 7,11-HD may be enough to establish a species barrier, the effect of this compound is moderated by the relative quantity of other signals. Indeed, the effects of 7,11-HD are particularly noteworthy because it functions as an attractant in an intraspecific context, whereas in an interspecific context, it aids in species recognition, thereby placing social communication and speciation on the same continuum.

The logic of pheromonal communication in *Drosophila* seems to be based on a foundation that imparts general attractiveness to a fly (Online Supplementary Figure. 3). In our study female oenocytes are the primary organ for communicating species and sex identity to males. Others have shown that males use species-specific acoustic tags within their love song for females during courtship (Cowling and Burnet, 1981; Kyriacou and Hall, 1986; Wheeler et al., 1991). Thus, both acoustic and pheromonal tags establish a context for social interactions by regulating sex and species recognition. Given that
individual flies regulate their own hydrocarbon display in accord with their social surroundings (Kent et al., 2008), it is plausible that these compounds also function in individual recognition.

3.3 METHODS

Full Methods and any associated references are available in the online version of the paper at [www.nature.com/nature](http://www.nature.com/nature).
3.4 CHAPTER 3 FIGURES

Figure 3.1 Oenocytes produce cuticular hydrocarbons. A. Tissue-specific expression of desat1 type E isoform in the adult oenocytes. RT–PCR on desat1 transcripts from different adult tissues. Transcript-specific primer combinations were used to detect desat1 isoforms A–E. Gene-specific primers for desat1 and rp49 were used as controls. B. Organization of the desat1 locus. Black boxes, non-coding exons; orange; coding. A–E are alternative promoters. C. PromE(800)-gal4 driver recapitulates desat1-E isoform expression. PromE(800)-gal4 driving UAS-nuclear GFP expression in adults is shown. AcGL, accessory glands; Ej. bulb, ejaculatory bulb. D. Dorsal abdominal fillet preparations of control (PromE(800)-gal4, TubP-GAL80ts/UAS-nuclearGFP) and oenocyte-ablated (oe-) females (PromE(800)-gal4, TubP-GAL80ts/UAS-hid, UAS-nuclear GFP) demonstrating the loss of GFP expression after cell-death induction. Scale bar, 200 μm. Weak fluorescence in oe-animals comes from necrotic tissues devoid of nuclear structures (inserts magnified in lower panels: scale bar, 20 μm). E. Haematoxylin and eosin staining of abdominal fillet as in D. demonstrating disruption of the oenocytes in oe- flies. Other abdominal tissues appear normal. fb, fat bodies; m, muscles (arrowhead); oe, oenocytes (indicated by asterisks, form a strip strapped under the dorsal abdominal muscles); t, trachea. Scale bars, 100 μm. F. Cuticular hydrocarbons of single flies were analyzed using gas chromatography. Chromatograms plot the peak area associated with the amount of a specific hydrocarbon, scale is in millivolts (mV). G. Mean sum of cuticular hydrocarbons (Σ CHs).Genotypes as in D. Hydrocarbon quantification broken down by chemical classes. Error bars indicate s.e.m. n = 20,21. Online Supplementary Tables 1 and 2 show values for individual compounds and for additional control genotypes.
Figure 3.2 | Hydrocarbons regulate sexual attractiveness. A. Sexual behaviour of oe- and control (ctrl) males with wild-type (WT) virgin females. Courtship indices are not significantly different ($p = 0.30$). n = 27, 38 for courtship index and 158,148 for mating latency. B. Male–male interaction of oe- males paired with wild-type or oe- males. oe- males labelled (17-T) were treated with approximately 1 mg of synthetic 7-T. Bars labelled by the same letter (a or b) in the histograms are not statistically different (ANOVA, $p < 0.01$; n = 10–19). C. Behaviour of wild-type males towards oe- females. n = 38, 39 for courtship index and 139,146 for mating latency. D. Competitive mating assays in which oe- females or males were pitted against a control animal of the same sex for copulation with a wild- type male or female. The preference index is the relative advantage of oe- animal over the control. n = 70, 15. E. oe- virgin females treated with increasing amounts of synthetic 7,11-HD. Females were paired with wild-type males and the time between introduction to mating initiation was recorded. n = 11–12. F. Control and oe- virgin females treated with increasing amounts of synthetic cVA. Treated females were paired with wild-type males and assayed as in E. Asterisks indicate significant differences between genotypes as determined by a two-tailed Student’s t-test (**$p < 0.01$). There was a significant dose effect (ANCOVA, $F_1, 191 = 125.27, p = 0.001$) and dose by genotype interaction (ANCOVA, $F_1, 191 = 9.0, p = 0.003$). n = 22–29. G. Control and oe- virgin females treated with cVA and assayed as in F. All oe- females were treated with approximately 400ng of 7,11-HD. No significant differences were observed between genotypes and there was no significant dose by genotype interaction (ANCOVA, $F_1,176 = 2.2, p = 0.14$), but the dose effect remains (ANCOVA, $F_1,176 = 90.1, p < 0.001$). n = 11–37. Error bars indicate s.e.m.
Figure 3.3 | 7,11-HD regulates courtship between Drosophila species. A. Phylogenetic relationships of the four Drosophila species used in this study, along with a timescale for evolution in this group (Russo et al., 1995). Partitioning of the four major female hydrocarbon compounds (7,11-HD; 7,11-ND; 9,23-TTCD, (9Z, 23Z)-tritriacontadiene) in these different species (Jallon and David, 1987): light grey indicates absence, grey indicates minor amounts, and black indicates a major fraction of the total hydrocarbon. B. Wild-type males courtship behaviour in intra- and interspecific pairings with females of the indicated species and genotype. n = 10–19. C. Wild-type D. melanogaster male courtship towards females from the indicated species. n = 10–12. Females labelled (17,11-HD) were treated with approximately 500 ng of synthesized 7,11-HD. Error bars indicate s.e.m. Bars labelled by the same letter (a, b, c or d) in the histograms are not statistically different (ANOVA, p < 0.01).

Figure 3.4 | Mating between D. melanogaster females and D. simulans males is prevented by 7,11-HD. A. Percentage of females of the indicated species and genotype that mated with D. simulans males in a 24-h period. n = 18–48. B. Percentage of D. simulans or oe- D. melanogaster females that mated with D. simulans males in a 12-h period. Females were treated with increasing amounts of synthetic 7,11-HD. Single virgin females were paired with single D. simulans males. The fraction of pairs that mated within 12 h is reported. Asterisks indicate significant differences between genotypes as determined by a chi-squared test (**p<0.01). n = 10–25. C. oe- females treated with increasing amounts of synthetic 7-T and paired with D. simulans males as in b. One group of oe- females was treated with approximately 200 ng of 7,11-HD in addition to 7-T. n = 19–37.
Chapter 4  GENETIC SCREEN PARADIGM

4.1 OBJECTIVE AND INVESTIGATION STRATEGY

The findings detailed in CHAPTER 1 and CHAPTER 2 pioneer our mechanistic understanding of social influence on D. melanogaster behaviour. Most importantly, they highlight the integral role that circadian clocks play in mediating social influence, whereby clocks of both ‘interacter’ and ‘interactee’ communicate to generate coordinated behaviours. As mentioned previously, these findings point to the genetic and neural substrates involved in the manifestation of social influence under various conditions. Yet, a great deal remains to be uncovered regarding the mechanisms by which individuals send, receive, analyse, and respond to these social cues. Thus, the identification of additional genes is necessary for building more comprehensive models.

The aim of the rest of this thesis is to identify additional genes underlying social interactions that affect the clock mechanism in D. melanogaster. More specifically, investigations focus on mechanisms within the interaction. My research investigates the emitter fly responsible for inflicting social influence on a companion. Probing for substrates influencing specifically the clock mechanism in a recipient is highly promising, since many investigations have recurrently emphasized the circadian system as highly responsive to the social environment (Fujii et al., 2007; Krupp et al., 2013; Krupp et al., 2008; Levine et al., 2002a). The conducted investigations employed both forward and reverse genetics. Both approaches were carried out using the same genetic screen discussed in the following chapter.

4.2 SCREEN DESIGN AND IMPLICATIONS

Screen Design. The screening assay consists of a dyadic social context in which two males are placed together in one well of a 96-well plate system, and monitored for five days. One of the flies (focal) is a real-time PER luciferase reporter containing a BG-luc transgene (Stanewsky et al., 1997), whereas the other fly (stimulus) is the mutagenized individual (FIGURE 4.1 D).

The BG-luc reporter was originally developed to monitor the expression of PER. It contains a transgene in which the per promoter and two thirds of the PER coding sequence were fused to cDNA encoding firefly luciferase (FIGURE 4.1 A). This transgene acts as translational and post-translational PER marker. When provided with food media fortified with luciferin substrate and monitored using a TopCount scintillation detector (TC), an oscillating bioluminescent signal is generated (FIGURE 4.1 B & C). The
luciferase mediated bioluminescence serves as a marker for PER protein presence in the reporter. The bioluminescent activity of luciferase is suitable for measuring the oscillation of PER. It has a relatively short half-life, which allows fine time resolution of cycling. It can also be assayed continuously and non-invasively in many single animals. The amplitude, period, rhythm strength, and phase (defined below) of the  

*per*-controlled rhythmic luminescence characterize the collective status of all clocks in the reporter.

A male focal individual was used since males have been investigated the most as receivers of social signals, as was surveyed in Chapter 2. A mutant male stimulus companion was chosen on the other hand for practicality purposes. These included avoiding the use of females, whose progeny would for instance deplete the nutrient media at a higher rate. Several studies legitimized the choice of this MM social setting as discussed in Chapter 2. Briefly, males in an MM pair display locomotor activity patterns that are different from single males, implicating a social influence (Fujii et al., 2007). Social experience in an MM pair also modulates individual long-term locomotor activity, where the clock system of host and visitor are involved in the manifestation of such a social effect (Lone et al., 2011). Experience within an MM group also influences long-term locomotor activity, as well as modulates the expression of clock genes (Krupp et al., 2008; Levine et al., 2002a; Lone and Sharma, 2011b). The circadian system of visitors and hosts are also involved in the manifestation of this latter effect.

**Implications.** In the social context suggested, changes in the reporter’s circadian signal (focal) in the presence of a mutant (stimulus) in comparison to the presence of a WT individual (stimulus), permit the identification of novel genes in stimulus mutagenized subjects that affect PER expression in the reporter. Deviations in the reporter’s PER expression reflect changes in clock response to the presence of the mutagenized subject. Upon successful mapping of such mutations, component(s) underlying the social cue that is emitted from the mutagenized individual and detected by the recipient’s circadian clock mechanism would be uncovered. As a result, the two discrete ends of the molecular pathway through which the social experience is being manifested would be defined— the  

*per* gene product on the receiver’s end and the novel gene on the emitter’s end. Overlaying previous findings onto the outcome of this screen implies that the novel gene and the circadian system of the stimulus individual constitute 2 landmarks from which further investigations can question the mechanism with which a social signal is emitted. On the focal receiver’s end, olfaction as well as the time-keeping system constitute another 2 landmarks with which an individual receives, integrates, and responds to social information. Although defining an emitter and receiver is not a biologically sound distinction due the
feedback individuals are expected to inflict on each other; information extracted from such investigations is bound to expand our knowledge of the neurogenetic substrates that mediate social influence on behaviour under at least some conditions.

4.3 SCREEN OUTPUT MEASURES

Individual PER-driven bioluminescence counts per hour were first measured through the screening assay and plotted in time series format (Figure 4.1 C). Several aspects of rhythmicity in expression were subsequently quantified.

**Individual Mean Bioluminescence Counts.** This measure represents average intensity of bioluminescence over the monitored period within an individual, regardless of any cyclical properties. For instance averaging all the data points in the example time series shown in Figure 4.1 C would represent individual mean bioluminescence counts. Mean bioluminescence measures have also been calculated for the last 72, 48, and 24 hours. This was performed to maximize the detection of late occurring effects that might have been masked by earlier values. The possible occurrence of such late effects was anticipated since the influence of social experience does not always occur immediately as was discussed in section 2.4 (Krupp et al., 2008; Levine et al., 2002a; Lone et al., 2011).

**Individual Period.** This measure represents the time it takes to complete one full cycle of a repeated pattern. In this case, the pattern is bioluminescence. The example fly in Figure 4.1 C shows that it takes around 24 hours for a cycle to repeat. Period was calculated through autocorrelation (Levine et al., 2002b).

**Individual Rhythm Strength (RS) and Rhythmicity Index (RI).** These two measures serve the same purpose of quantifying the degree of pattern reproducibility across repeated cycles. The example fly in Figure 4.1 C shows that cycles i, ii, and iii have a relatively high degree of similarity. However, the areas marked with a diamond point to an example of differences that would decrease these RS and RI. Both measures are extracted from the correlation coefficient value corresponding to the third peak of an autocorrelation function (Levine et al., 2002b)

**Individual Phase Mean.** Phase refers to the average time at which peak bioluminescence occurs across repeated cycles. The asterisks shown in the example time series in Figure 4.1 C mark the time at which peak bioluminescence occurs within each cycle. Phase information was extracted using circular statistics (Levine et al., 2002b)
**Individual Phase Consistency.** Individual phase consistency reflects the variance in an individual’s mean phase (Levine et al., 2002b). The example bioluminescence time series in Figure 4.1C shows that between cycles, peak bioluminescence occurs at similar times.

### 4.4 Customized Automated Analysis

**Current Resources.** Data collection and analysis resources to handle this social screen have already been established (Levine et al., 2002b). These analyses were successfully used to isolate the cry* mutation in the cry locus by screening for arrhythmic bioluminescence in BG-luc mutant flies (Stanewsky et al., 1998), and identifying circadian-clock-regulated enhancers and genes using luc cDNA in a modified enhancer-trap mutagenesis (Stempfl et al., 2002). These available analysis resources were used for the screening of mutant lines in the social assay. Once a potentially significant effect was detected, the line was retested for further confirmation.

**Assay-Specific Shortfalls.** Re-examining the influence of candidate lines on the reporter’s PER expression as described earlier revealed that such effects are 1) subtle and 2) highly variable. This was the result of the small magnitude of effects, and mutant lines influencing their accompanying PER-reporter only in a subset of the conducted experiments. Furthermore, effects across experiments lacked consistency in directionality.

One reason for the subtlety of such effects might be that the screen was carried out in constant light-dark conditions, where light has strict control on the magnitude of observed changes. Another might simply lie in the nature of social influence on physiology, where subtle effects might be the biologically sensible expectation. Most importantly, all molecular clocks, including peripheral clocks, in the reporter are being monitored. Thus different clocks within one individual might not respond in the same manner/direction, decreasing the detection power of certain tissues’ or cells’ responses. If true, this possibility would also be compounded by the subtle and variable nature of responses.

These are unique difficulties that were not faced by previous studies, at least not to the same extent. Previous work investigated mostly two types of scenarios. The first involved examining the influence of treatments within the same individual, such as the influence of certain mutations on bioluminescence. The second examined the influence of strong external abiotic factors such as temperature and photoperiod. Due to the potency and direct nature of such manipulations, when an effect was detected it was usually consistently detected in all experiments, and one example
experiment averaging multiple individuals was usually published. In the case of the indirect social screen however, this would clearly not be possible as a result of the aforementioned high between-experiment variability.

**Solution.** Employing an average score system that uses the ratio of the experiments with an effect divided by that of total experiments was not feasible. This was due to the inconsistent directionality of observed effects. Consequently, it was necessary to normalize prior to averaging data across experiments to generate the most comprehensive representation possible. Although a common practice, analysis resources that normalize and average bioluminescence time series across experiments were not available since they weren’t previously needed. Hence, customized MATLAB scripts were developed to achieve this purpose.

4.4.a **SPECIFIC NORMALIZATION OF BIOLUMINESCENCE COUNTS**

Averaging luminescence counts/time across experiments is hindered by several irrelevant between-experiment differences. These differences increase overall variability in the data and can mask treatment-specific bioluminescence patterns. One example is the batch-specific characteristics of luciferin provided by the supplier, as certain batches seem to simply generate more bioluminescence than others. Another factor is the age of the luciferin-supplemented nutrient media. The luciferin-supplemented media appears to degrade with time, acquiring a green coloration, and this is correlated with a decrease in bioluminescence. When working with large scale experiments, it is unfortunately logistically not feasible to ensure that the luciferin-supplemented media is always the same age across all experiments. As a result, even when the concentration of luciferin is kept constant, the above irrelevant factors influence bioluminescence on a per-experiment basis. It therefore became necessary to normalize the data accordingly for each experiment before conducting across-experiment averaging.

As there was no existing normalization procedure to handle TopCount data, the following procedure was developed in order to process large scale experiments. The normalization process involves creating one average for all control flies within an experiment. All time-points/individuals within each experiment are then divided by their respective control mean. As a result, all values reflect deviations from the experiment-specific control mean. Furthermore, since all values are divided by one overall mean rather than the control’s mean at a specific time-point, cycling patterns are preserved in all treatments. While this normalization can control for between-experiment luciferin differences, it is
critical for accuracy that all luciferin-supplemented media used within an experiment is of the same age. MATLAB Code 1 was developed to carry out this normalization step.

4.4.b Alignment of Data from Various Experiments

After normalization of bioluminescence levels, experiments were aligned to the nearest time-point. This allowed comparison across experiments that were started at non-standardized times. This was necessary since it was not logistically feasible to have all experiment commence at the same time with no exception. MATLAB Code 2 was developed to carry out such an alignment.

4.4.c Quantification of Several Aspects of PER-Driven Bioluminescence

Individual Bioluminescence Counts per Hour Time Series. Bioluminescence counts were normalized to the controls on a per-experiment basis as described above (Section 4.4.a). Thus the values for each time-point represent deviations from the overall control value (Figure 4.2 A). The counts of individual replicates were averaged for each time-point.

Individual Mean Bioluminescence Counts. This measure was also based on normalized counts (Section 4.4.a). The same applies for mean bioluminescence calculated for the last 72, 48, and 24 hours. Individual mean counts for the time periods in question were averaged for all replicates (Figure 4.2 B, C, D, & E).

Individual Period. Calculation of each individual’s period was performed using MATLAB codes that conduct both autocorrelation (Figure 4.2 F) and maximum entropy spectral analyses (Figure 4.2 G) (Levine et al., 2002b). Individual periods were then averaged for all replicates.

Individual RS and RI. Both measures rely on autocorrelation analysis and are generated using previously developed MATLAB codes (Levine et al., 2002b). In this study, the average RS of a certain treatment was not calculated after averaging the bioluminescence time series of all individuals within a treatment. Doing so addresses the question as to whether individuals within a group are well synchronized to each other. If they are, pooling records would maintain a strongly rhythmic pattern. If they are not, individual patterns ‘cancel out’ decreasing overall rhythmicity. Since synchrony is not the question of interest, the RS and RI values were determined for each individual instead then averaged. Averaging such values addresses the rhythmicity of individuals within a treatment irrespective of group synchrony (Figure 4.2 H & I).
**Individual Phase Mean.** Phase has also been determined for each individual using previously developed MATLAB codes (Levine et al., 2002b). Since phase calculations might be influenced by experiment-specific variables, phase calculations have also been normalized on a per-experiment basis (Figure 4.2 J, L, M, N, & O). This aimed to optimize the detection of treatment-specific patterns upon averaging across experiments. This process involved setting the average phase of controls within an experiment to zero. Consequently, the phase of individuals is represented as deviations from that of the controls. MATLAB Code 3 and MATLAB Code 4 represent the previously established phase calculations and plotting codes (Levine et al., 2002b) edited to generate normalized estimations of phase.

**Individual Phase Consistency.** Individual phase consistency was also been determined for each individual using previously developed MATLAB codes (Levine et al., 2002b) (Figure 4.2 K, M, & O).

MATLAB Code 5 was developed to collect all the above described measures using previously established (Levine et al., 2002b), previously established and edited, or newly established MATLAB scripts. The script was created to handle large batches of experimental groups from large scale experiments. The output format facilitates subsequent analysis described below.

4.4.d **Addressing Inequality in Sample Sizes of Experimentals and Controls**

Due to the large number of experimental lines tested per experiment, the number of control flies included was always much larger than any single experimental line. Among other reasons, this was done as a precaution to avoid situations where a whole experiment is lost as a consequence of high death rate among the controls. This tactic however comes at price. Certain measures, especially that of phase are extremely sensitive to inequalities in sample size, and significance might be detected as a result of a high replicate number in one sample. To control for this inequality in sample size, a random sampling (bootstrapping) approach was developed. In this technique, a sample of size equal to that of experimentals was chosen from the controls. The measure of interest was calculated and stored. This process was repeated 9,999 times as recommended by the literature (Curran-Everett, 2009). Subsequently, the average of all the 10,000 stored measures was used as a reliable estimate.

The final bootstrapped time series record (Figure 4.2 A) or final mean and SEM for a specific variable (Figure 4.2 B to K) can then be used to compare the experimental group against while satisfying the need for equality of sample sizes. In the case of comparing the different measures, a standard t-test was used. MATLAB Code 6 was developed to bootstrap the time series analysis, MATLAB Code 7 was
developed to bootstrap the mean and SEM of controls and experimentals for all other measures. Both scripts were designed to accept batch experimental lines at once.

Although the bootstrapping approach was successfully applied to multiple measures (see list above), it is inappropriate for phase comparisons using circular statistics (Levine et al., 2002b). Thus, while phase comparisons were possible by using datasets combined across experiments, they suffered from unequal sample sizes when the circular statistics method was employed (Figure 4.2 N & O). For illustrative purposes, phase comparisons were also conducted using equal sample sizes by sampling one random subset of individuals from the treatment with the larger sample size (Figure 4.2 L & M). This subset size was chosen to be equal to the treatment with the lesser replicates. Phase comparisons were performed both with (Bivariate (Figure 4.2 M & O) and without taking phase consistency within an individual into account (Figure 4.2 L & N) (Levine et al., 2002a).

4.4.e CAVEAT TO USING INDIVIDUAL FLIES AS REPLICATES & SOLUTION

While it is standard and accepted practice to treat individuals as replicates, when combining across experiments, a potential problem arises when the number of individuals across experiments is not consistent. If there were any experiment-specific effects, then experiments with a larger number of individuals would proportionally over-influence the total replicate pool. To minimize potential false positives detected by using individuals as replicates, MATLAB Code 8 and MATLAB Code 9 were developed to analyze the same datasets using experiments as replicates (technical replicates), and to generate the relevant means, SEMs, and p-values (Figure 4.3). However, since experiment numbers are typically low compared to the number of individuals, interesting effects could sometimes fail to reach significance, but would otherwise with a higher number of experiments. For these reasons, measures were analyzed using both individuals and experiments as replicates. Both approaches were always consulted before reaching any major conclusions to achieve a compromise between maintaining moderate screening throughput and minimizing false negatives/positives.

4.4.f AUTOMATED ANALYSIS

The preceding analysis has been automated in a manner that allows the user to generate all the necessary measures, statistical analyses, and associated graphs through MATLAB. This includes the newly developed analysis methods and plots. Such automation reduces the hands-on time spent analyzing a full screening experiment (~80 lines) from days to a couple of hours.
4.5 Screen Suitability

Before screening for mutations that influence PER expression in a reporter, it was first necessary to determine the ‘WT’ PER response, if any, to companionship. To that end, PER expression of a solitary reporter was compared to that of a reporter housed with a WT individual (Figure 4.4 & Figure 4.5). Most aspects of rhythmic PER expression were unaffected by the presence of a companion except for amplitude. Overall PER expression decreases in the presence of a companion by about 10% (Figure 4.4 A, B, C, D, & E), and this decrease seems to occur gradually (Figure 4.4 A).

Albeit through a different social manipulation, Krupp et al. (2008) also observed a decrease in clock gene expression in the head and oenocytes of individuals from a mixed group, compared to those housed in a genotypically homogenous group. A decrease in *tim, per*, and *Clk* transcription was detected in the oenocytes, whereas a decrease in *per* and *Clk* expression was observed in the heads. Consequently, the social screen successfully captures socially mediated changes to circadian PER expression, and it was deemed appropriate for the dissection of mechanisms underlying social influences on clocks.

On a technical note, it is worth noting that as has been discussed in Section 4.4.E, examination of the results was carried out using flies as replicates with random resampling (Figure 4.4), as well as with experiments as replicates (Figure 4.5). As can be observed, significance in the decrease of overall bioluminescence was only detected when using flies (Figure 4.4 B TO E) and not experiments as replicates (Figure 4.5 B TO E). However a trend is clearly visible in the latter case and would likely approach significance with a higher number of experiments. This is a good example that justifies the random resampling approach mentioned in Section 4.4.D in conjunction with consulting technical replicates. Using this approach is a compromise between minimizing false negatives/positives while maintaining moderate throughput.

With the established suitability of the paradigm and an extensive automated analysis protocol, the screen was employed in a forward and reverse genetic manner. Both aimed to identify loci within the *Drosophila* genome that underlie the modulation of PER expression in a companion.
4.7 CHAPTER 4 FIGURES

**Figure 4.1 | Screen paradigm description.** A. The reporter individual (shown in green) harbors a BG-\textit{luc} transgene in which the \textit{per} promoter and two thirds of the PER coding sequence are fused to cDNA encoding firefly luciferase. This transgene acts as a translational and post-translational marker of PER. B. Luciferase catalyzes the conversion of luciferin to oxyluciferin emitting bioluminescence as a by-product. C. When provided with nutrient media fortified with luciferin substrate and monitored using a TC, BG-\textit{luc} transgenic flies emit an oscillating bioluminescent signal. The luciferase-mediated bioluminescence serves as a marker for the endogenous PER levels in all of the reporter’s PER-expressing tissues. The displayed example trace has a period of 24 hours since it takes around 24 hours for a cycle to repeat. Due to similarities in the patterns of each of the cycles, a high RI is predicted. Areas marked with a diamond point to example differences that would decrease RI. The asterisks mark the time at which peak bioluminescence occurs within each cycle (phase). D. The screening assay consists of a simple social context in which two individual reporters are placed together in one well of a 96-well plate system, and monitored for five days. The accompanying fly is either a mutagenized individual (right) or a wild-type control individual (left). The pattern of the reporter’s \textit{per}-controlled rhythmic luminescence in the presence of a mutant individual is then compared to that in the presence of a wild-type control.
Figure 4.2 | Example figure illustrating the customized and automated analysis of PER-driven bioluminescence using flies as replicates. All plotted measures have been defined and discussed in Section 4.3. The generation and analysis of all included measures have been performed as described in Section 4.4. The circular plots displayed in L to O have been performed according to (Levine et al., 2002b). L & N. Dif represents phase difference in hours. Asterisks represent controls whereas circles represents the treatment line at hand. Numbers in parentheses indicate the number of replicate flies within a treatment. pD is a p-value for comparing differences in the distribution of within treatment phase values. pM is a p-value for comparing differences between average phase of treatments. M & O. phi represents average phase of a treatment. r represents average phase consistency of a treatment. p is a p-value resulting from the bivariate comparison of both groups taking phase and phase consistency into account.
Figure 4.3 | Example figure illustrating the customized and automated analysis of PER-driven bioluminescence using individual experiments as replicates. All plotted measures have been defined and discussed in Section 4.3. The generation and analysis of all included measures have been performed as described in Section 4.4.
Figure 4.4 | Analysis of PER-driven bioluminescence of solitary reporter compared to a reporter co-housed with a wild-type conspecific using flies as replicates. All plotted measures have been defined and discussed in Section 4.3. The generation and analysis of all included measures have been performed as described in Section 4.4. The circular plots displayed in L to O have been performed according to (Levine et al., 2002b). L & N. Dif represents phase difference in hours. Asterisks represent controls whereas circles represents the treatment line at hand. Numbers in parentheses indicate the number of replicate flies within a treatment. pD is a p-value for comparing differences in the distribution of within treatment phase values. pM is a p-value for comparing differences between average phase of treatments. M & O, phi represents average phase of a treatment. r represents average phase consistency of a treatment. p is a p-value resulting from the bivariate comparison of both groups taking phase and phase consistency into account.
Figure 4.5 | Analysis of PER-driven bioluminescence of solitary reporter compared to a reporter co-housed with a wild-type conspecific using individual experiments as replicates. All plotted measures have been defined and discussed in Section 4.3. The generation and analysis of all included measures have been performed as described in Section 4.4. \( p=\text{NaN} \) indicates that it is not possible to perform statistical comparisons when only one replicate is available.
Chapter 5  FORWARD GENETIC APPROACH

5.1 MUTAGENESIS

Mutagen Choice. The mutagen employed was ethane methyl sulfonate (EMS). It is an alkylating agent that causes point mutations randomly within the genome (Ashburner et al., 2005). EMS has been proven to be effective in identifying a number of behavioural mutations, such as those in circadian clock genes (Konopka and Benzer, 1971; Stempfl et al., 2002). In addition, it is characterized by low toxicity to the flies when ingested at moderate concentrations (Ashburner et al., 2005). Non-chemical methods of mutagenesis such as the use of p-transposable elements would have also been feasible. However, the effects of p-elements are mostly disruptive, decreasing the probability of hypomorphic and gain of function mutations. Additionally, given that p-elements have preferential genomic insertion sites (Rosato, 2007), this method is not entirely random. On the other hand, choosing chemical over p-elements mutagenesis is coupled to a higher difficulty in mapping anonymous mutations.

Dosage. In terms of dosage and administration, the standard mutagenesis protocol was followed (Ashburner et al., 2005; Ashburner et al., 2000; Greenspan, 2004; Rosato, 2007). 15 mM of EMS in a 1% sucrose solution was used. Owing to its instability in aqueous solutions, EMS quality varies with stocks and/or lab conditions (Ashburner et al., 2005). As a result its mutagenic efficiency was monitored by scoring for X-lethal mutations (Rosato, 2007) (Figure 5.1). The aim was to attain 20 to 30% lethality. At this rate, a fair compromise between high exposure and low lethality is attained (Rosato, 2007). The highest concentration that produced 20 to 30% lethality while maintaining an acceptable number of successful lines was 15mM (Figure 5.1 B).

Mutant Lines Establishment. The sex chromosome was evaluated for mutations that cause an effect on the reporter’s response to a companion. Figure 5.3 displays the crossing scheme used to generate the individual mutant lines. It is noteworthy that such lines continued to carry mutations on the 2nd and 3rd chromosomes. Thus the 2nd and 3rd chromosomes were also examined. However, due to the heterozygosity of these mutations in the established lines, the necessary measures were taken when needed to handle this appropriately, as discussed below and shown in Figure 5.2, Figure 5.5, and Figure 5.6.
5.2 Screening and Partial Mapping.

2552 lines were generated (Figure 5.3) and screened (Figure 5.2 Steps 1 & 2), of which 14 lines caused deviated PER responses in the co-housed reporter, compared to a reporter’s PER response in the presence of a control companion. A series of crosses were employed to isolate the X, 2nd, and 3rd chromosome from each of the 14 potential mutant lines (Figure 5.2 Step 5). This was conducted in an effort to identify the chromosome on which the mutation of interest resides in each of the lines. As a result, 36 descendant lines were generated (14 lines x 3 chromosomes - 6 failed crosses). The crosses employed to isolate mutagenized X chromosomes are shown in Figure 5.4. The crossing schemes employed to isolate mutagenized 2nd and 3rd chromosome mutations are shown in Figure 5.5. As seen in this latter figure, the crosses used to isolate mutagenized 2nd and 3rd chromosomes began with heterozygous individuals, due to crosses used to generate these individuals in the first place (Figure 5.3). As a result, although the outcome of crosses in Figure 5.5 was lines with mutations on one of the autosomes only, such mutations were not in a homozygous state. This heterozygosity for interesting mutations creates mapping problems since mapping requires commencing with a true breeding line for the mutation in question. For this reason, this problem was further addressed and is discussed later.

Each of the 36 descendant lines were then individually re-tested (Figure 5.2 Step 6). For certain lines, their influence on the reporter’s PER-driven bioluminescence significantly diminished or ceased to exist in the descendant lines. As an example, this can be seen when the RI of parent line 1414 was compared to the RI of its 1st, 2nd, and 3rd isolated chromosome descendants - lines 1414-X, 1414-2nd, and 1414-3rd respectively (Figure 5.7 A). This occurred possibly due to the crossing scheme used to establish the parent lines (Figure 5.3), where the original phenotype could have been the result of an interaction between two or more mutations on separate chromosomes. Subsequently, when each of the chromosomes were separated into different lines, the phenotype disappeared. This could also explain why certain descendant lines displayed a new phenotype that was not originally present in the parent line. In such cases the presence of mutations on one chromosome might have masked other mutations in the parent line. An example of such a case can be seen in Figure 5.7 B, where line 86-2nd displayed a phase shift that was not detected in the parent line 86. For other lines, their influence on the reporter’s PER-driven bioluminescence persisted. As an example this can be seen when the mean counts/hr. of parent line 192 was compared to all of its 1st, 2nd, and 3rd isolated chromosome descendant lines (Figure 5.7 C).
In total, 10 descendant lines were chosen for further investigation, 2 lines with mutations mapping to the 2\textsuperscript{nd} chromosome, 4 lines to the 3\textsuperscript{rd}, and 4 lines mapping to the X chromosome (TABLE 5-1). Grouped otherwise, 6 descendant lines produced a phase shift in the reporter’s rhythmic PER expression compared to a reporter in the presence of a control. 4 lines caused a change in the reporter’s overall PER expression levels, and 2 lines imposed a change in the reporter’s RI. Out of these, 2 lines produced 2 simultaneous phenotypic changes in the reporter. Line 298-2\textsuperscript{nd} produced a phase shift and an increase in RI, whereas line 298-3\textsuperscript{rd} caused a phase shift as well as a decrease in overall expression levels. The phenotypes of the candidate lines are summarized in Table 5-1. Since the original crossing scheme was mainly designed to screen for X chromosome mutations, lines with mutations mapping to the X chromosome were of particular interest. One challenging consequence was that the screen was conducted with males and hence complementation tests were not possible. This was the case since males carry one copy of the X chromosome while complementation tests require 2 homologues of the chromosome in question. One alternative would have been to re-establish the screen phenotype using females harboring two X chromosomes, and carrying out the complementation tests. However other investigation steps were prioritized.

Mutations mapping to the autosomes were also of interest. However, as mentioned previously, although the descendant lines generated (FIGURE 5.2 STEP 5 AND FIGURE 5.5) were lines with mutations on one of the autosomes only, such mutations were not in a homozygous state. This heterozygosity creates mapping problems since it is necessary to start with a true breeding line for the mutation in question. For this reason, additional crosses were carried out to extract around 20 chromosomes from each of these lines and establish true breeding lines (FIGURE 5.2 STEP 7 AND FIGURE 5.6). Ideally, a subset of the 20 lines generated from each of the autosomal putative mutants would be homozygous for the autosomal mutation in question, as opposed to homozygous for a WT chromosome. These lines have been generated (6 autosomal putative lines x 20 descendants) and are being maintained in the lab for future screening. This was performed in anticipation of detecting the phenotype of the original autosomal mutant parent line in one of these true breeding lines.

In usual circumstances, one or 2 candidate lines would be chosen for higher resolution mapping. However, the frequency of instrumental challenges faced with the TC was too high for high throughput phenotyping of recombinants. Furthermore, after extensive analysis of the putative mutants, it was concluded that TC screening required at least 3 experiments per line for conclusive phenotyping. For this reason, it became worthwhile to conduct a few secondary investigations prior to mapping. On one
hand, this would uncover more information about the mutations at hand. On the other hand, such investigations might reveal that other assays could be logistically more practical for mapping. As a result, some secondary characterization of the candidate lines was performed prior to mapping.

5.3 Secondary Characterization of Candidate Mutant(s)

Several secondary characterization assays were chosen to investigate further abnormalities associated with the mutations in the candidate lines at hand. This was done in an effort to understand the cells, tissues, and/or behaviours affected by the mutation, and through which the influence on the reporter might be manifested. Since the screening assay selected for mutations in a companion that alter PER expression in the reporter, it was reasonable to question the clock status of the mutants themselves. Locomotor activity is a clock-controlled behaviour and was one of the assays employed for secondary characterization. Clock gene expression as well as clock-related brain circuitry were also chosen for examining the candidate lines for any clock-related abnormalities. The potentiality of these investigations is further supported by previous findings. Clock mutants with abnormal locomotor activity have been shown to exert an influence on the locomotor activity and clock gene expression of co-housed WT conspecifics (Krupp et al., 2008; Levine et al., 2002a). Another possible scenario would be that the candidate lines might have altered phenomenal profiles through which the influence on the reporter is communicated. Several findings rationalize this line of thought. Olfactory cues have been found to mediate the influence of clock mutants on the locomotor activity of accompanying conspecifics (Levine et al., 2002a). Furthermore, the expression of the hydrocarbon synthesis enzyme DESAT, as well as several major hydrocarbons were found to respond to this social treatment (Krupp et al., 2008). These findings were also correlated with a change in mating behaviour. Consequently, this indirectly raises the question as to whether these candidate lines exhibit normal courtship and mating behaviour. Some of these secondary screens have already been completed while others will be completed post defense or in the future by other lab members.

5.3.a Locomotor Activity

i. Locomotor Activity Analysis

A significant portion of the locomotor activity analysis tools have already been established (Levine et al., 2002b). However, these tools are limited in certain aspects. In one respect, the batch analysis of a large number of lines can be tedious, time consuming, and does not include the necessary statistics
for all the measures considered. This limitation has been resolved by editing existing MATLAB scripts and the generation of new scripts for batch analysis, to produce all the necessary graphs in appropriate formats and including the relevant statistics. Furthermore, the available analysis kit does not extract certain measures such as day activity, night activity, ratio of night to day activity, amplitude of morning peak, evening peak, and ratio of evening to morning peak. This has also been addressed by generating scripts that extract these measures, plot them, and include the necessary statistics. Finally, the approach used in analyzing certain measures has been slightly altered from the existent analysis kit. For instance, when examining the estimated average period of a certain line, the existent scripts pool the activity of all replicates followed by period estimation. Consequently, the variability of periodicity within a line is not captured in a way that permits statistical analysis in period comparison between strains. Thus the existent scripts have been slightly modified to generate a period value for each individual, and an average was then subsequently calculated.

The locomotor activity analysis was designed to examine the following list of measures. Figure 5.8 will be used as a reference example. Each measure has been determined for each individual replicate then an average calculated for each line.

**Mean Activity Counts/Min Time Series (with Error Bars).** The counts of individual replicates were averaged for each minute. Error bars represent SEMs (Figure 5.8 A1).

**Mean Activity Counts/Min Time Series (without Error Bars).** The counts of individual replicates were averaged for each minute. Error bars have been omitted for improving the visualization of differences in activity pattern (Figure 5.8 A2).

**Mean Daytime Activity Counts.** Total daytime counts (ZT or circadian time (CT) 0 to 12) were summed and divided by the number of days (Figure 5.8 B).

**Mean Nighttime Activity Counts.** Total nighttime counts (ZT or CT 12 to 23) were summed and divided by the number of nights (Figure 5.8 C).

**Mean Morning Peak Activity Counts.** Total morning peak counts (ZT or CT 18 to 6) were summed and divided by the number of morning peaks (Figure 5.8 D).

**Mean Evening Peak Activity Counts.** Total evening peak counts (ZT or CT 6 to 18) were summed and divided by the number of evening peaks (Figure 5.8 E).
Mean Ratio of Nighttime/Daytime Activity Counts. Nighttime activity count was divided by the daytime activity count (Figure 5.8 F).

Mean Ratio of Evening Peak/Morning Peak Activity Counts. Evening peak activity count was divided by the morning peak activity count (Figure 5.8 G).

Mean 24Hr Activity Counts. Total 24 hour activity counts (ZT or CT 0 to 23) were summed and divided by the number of days (Figure 5.8 H).

Mean Period. Period was previously defined in Section 4.3 In this case however, it is mentioned in the context of activity instead of bioluminescence. Calculation of period was performed using MATLAB codes that conduct autocorrelation analysis (Levine et al., 2002b) (Figure 5.8 I).

Mean RS and RI. These two measures have been previously defined in Section 4.3 Both measures rely on autocorrelation analysis and were generated using previously developed MATLAB codes (Levine et al., 2002b) (Figure 5.8 J and K).

Mean Phase. Phase has been also previously defined in Section 4.3 and has been determined using previously developed MATLAB codes (Levine et al., 2002b). Phase was graphically represented using a bar graph (Figure 5.8 L), as well as circular plots (Figure 5.8 N & O). In the latter, the average phase of controls was set to zero so that the phase of individuals is represented as deviations from that of the controls.

Mean Phase Consistency. Phase consistency has been also previously defined in Section 4.3 and calculated using previously developed MATLAB codes (Levine et al., 2002b). Phase consistency was graphically represented using a bar graph (Figure 5.8 M), as well as circular plots (Figure 5.8 O).

As mentioned earlier, MATLAB Code 10 and MATLAB Code 11 were developed to collect all the above described measures, perform statistical analysis, and plot all measures. The output consists of one large figure in which each line is compared to the appropriate control for all measures as shown in the example Figure 5.8.

ii. Locomotor Activity of Putative Mutants

The locomotor activity phenotypes of the candidate lines are summarized in Table 5-2 and are documented in detail in Appendix II. Of particular interest were 2 lines with mutations mapping to the X
chromosome whose locomotor activity phenotypes in LD and DD were strikingly similar. These lines were 192-X and 1414-X. Similar to the reduction in PER expression they inflicted on the reporter in the screening assay, the locomotor activity of these lines was significantly reduced by 70 and 77% in LD and 52 and 61% in DD respectively (Table 5-2, Figure 5.9, Figure 5.10, Figure 5.11, & Figure 5.12 B, C, D, E, &H). This reduction was the result of decreased day and night activity. Furthermore, both lines displayed a phase shift in locomotor activity by around 1.5 hours in LD and not DD (Table 5-2, Figure 5.9, Figure 5.10, Figure 5.11, Figure 5.12 L, N, & O). Mean phase consistency of lines 192-X and 1414-X on the other hand, was higher by 14 and 12% in LD respectively (Table 5-2, Figure 5.9, Figure 5.10 M). In DD however, line 192-X’s phase consistency was also higher by 21%, whereas that of line 1414-X was not significantly different (Table 5-2, Figure 5.11, & Figure 5.12 M). Since line 192-X was an overall healthier line, it was chosen for further mapping and characterization.

It is worth noting that not all mutant lines displayed locomotor activity abnormalities as was observed in lines 192-X and 1414-X. While line 1036-X exhibited significant differences from the control in several measures in LD, it did not display any difference from the control in DD (Table 5-2, Figure A.37 and Figure A.38).

Lines with mutations mapping to the autosomes also exhibited significant differences from the control when considering several locomotor activity aspects. This can be seen when examining the locomotor activity of lines 298-2nd and 86-2nd (Table 5-2). While line 298-2nd exhibited the most differences in night activity in LD (Figure A.35 and Figure A.36), line 86-2nd displayed reduced day and night activity levels in LD, reduced night activity in DD, as well increased rhythmicity strength in DD (Table 5-2)(Figure A.33 and Figure A.34).

5.3.b CLOCK GENES EXPRESSION

Since locomotor activity is clock-controlled, examining whether the candidate lines displayed normal clock gene expression was a question of interest. For this reason the BG-luc transgene was crossed into the background of line 192-X and its corresponding control, since 192-X since was the line chosen for recombination mapping. This manipulation permits examining PER levels in the mutant line by monitoring per-promoted bioluminescence. The same was performed with tim-luc in order to monitor tim expression. The crosses involved in creating these lines are shown in Figure 5.14. These lines have been generated and are now available in the Levine laboratory for future testing post-defense or by
other lab members. One round of TC monitoring will provide sufficient insight into whether this candidate line exhibits normal PER and \textit{tim} expression.

5.3.c \textbf{Clock-related Circuitry}

Given the PER expression influence the candidates cause in the accompanying reporter, and the corresponding locomotor activity phenotypes, it was sound to question whether the number of clock-related brain neurons and their fasciculation is abnormal or not. The neurons chosen for this investigation were \textit{Pdf}-positive neurons due to their crucial role in normal locomotor activity (Hardin, 1994). PDF signaling has also been implicated in synchronizing physiological and behavioral processes that govern social interactions (Krupp et al., 2013). Thus, \textit{Pdf-gal4} and \textit{UAS-gfp} were crossed into the background of the line 192-X and its control. Brains were subsequently dissected and imaged using a GFP dissecting microscope (Figure 5.15). Spatial projection of \textit{Pdf-gal4} expressing neurons reported by \textit{UAS-mCD8::GFP} in dissected 192-X brains was similar to WT for both the s- and l-LN\textsubscript{s}. In both 192-X and WT controls, the s-LN\textsubscript{s} projected to the dorsal region of the brain. In addition, the l-LN\textsubscript{s} of both lines projected to the optic lobe as well as to the contralateral LN\textsubscript{s} and optic lobe through the posterior optic tract.

5.3.d \textbf{Hydrocarbon Profiles and Expression of the Hydrocarbon Synthesis Enzyme DESAT}

As mentioned in the beginning of Section 5.3, it is plausible that the candidate lines might have altered phenomenal profiles through which the influence on the reporter is communicated (Kent et al., 2008; Krupp et al., 2008; Levine et al., 2002a). Cuticular hydrocarbons from 192-X males and the corresponding control were extracted, processed through Gas Chromatography, quantified, and analyzed statistically. This was performed through a collaboration with a master’s student in the Levine Laboratory who performed the extraction portion of the process. Indeed, the hydrocarbon profile of line 192-X males was significantly different from that of the corresponding control (Table 5-4). An overall reduction of 25% was observed when considering total hydrocarbons. However, hydrocarbon classes and hydrocarbons within each of the classes did not display similar reduction magnitudes. More specifically, although alkanes exhibited a 30% reduction in total amounts, nC29 in line 192-X was 32% higher than in the corresponding control line. Almost all of the remainder alkanes were significantly reduced, with the shorter chain alkanes, nC22, nC23, and nC24 displaying the severest reduction (-37, -35, -44% respectively). Similar to alkanes, monoenes (hydrocarbons with a single double bond) were also reduced by 30% in line 192-X. With the exception of two short chains monoenes that were not
significantly different, all other monoenes showed a similar pattern of reduction. Methyl alkanes on the other hand, exhibited a unique pattern of differences from the control line. The shortest and longest methyl alkanes, 2MeC22 and 2MeC30 respectively, were not significantly different from the control line. The two 2nd shortest methyl alkanes, 2MeC24 and 2MeC26 were found reduced by 49% and 21% respectively. The 2nd longest methyl alkane 2MeC28 on the other hand was 23% more abundant in line 192-X. Since not all methyl alkanes displayed the same directionality in differences from the control, the overall difference in methyl alkanes was only 9%. The compound that displayed the most striking difference from the control line was cVA. Line 192-X expressed more than double the amount of cVA found in the control line (139% increase). The striking magnitude of this increase was not observed in any of the other compounds.

Since DESAT is a well-known enzyme involved in hydrocarbon synthesis, and whose expression has been shown to respond to the social environment (Kent et al., 2008), it was hypothesized that its expression in line 192-X might be different from that of the control. Thus the necessary crosses to insert desat-luc into the background of line 192-X and its control were carried out (Figure 5.14). This permits real time examination of desat-promoted luminescence in this mutant, providing in vivo data on desat expression. These lines have been generated and are now available in the Levine laboratory for future testing post-defense or by other lab members.

5.3.e COURTSHIP AND MATING BEHAVIOUR

Since courtship and mating behaviour are highly responsive to the social environment both in a group and pair setting (Fujii and Amrein, 2010; Fujii et al., 2007; Krupp et al., 2008), it was also interesting to examine whether the candidate line 192-X displayed normal courtship and mating behaviour. Males were monitored with females over a 10 minute time period using LIFESONG software (Bernstein et al., 1992). Courtship analysis was conducted as described in the Methods Chapter Section 9.5.

Line 192-X displayed similar courtship characteristics to its control in all respects except for courtship index, which was significantly less (Figure 5.16). Since percent mated and mating latency were not significantly different from the control, this implied that 192-X males mated within the normal time frame, yet simply spent less time courting. Interestingly, levels of wing extension and attempted copulation carried out within the time invested in courtship were maintained at WT levels as well. Such a courtship profile indicates that although this line courts less on average, it is still able to successfully
mate within the WT time frame. Yet, instead of successfully mating faster, these males simply spend less effort courting to achieve the same results a WT male achieves.

5.4 **RECOMBINATION SNP MAPPING**

As previously discussed, given the instrumental instability of the TC, and the number of replicates required to confirm the phenotype of a specific line, another assay was chosen for phenotyping the generated recombinants. Locomotor activity was the assay of choice due to its high throughput and reliability. A downside to this approach is that the locomotor activity phenotype does not necessarily have to fully correlate with the phenotype inflicted on the companion reporter. This is especially the case since it is possible to have more than one mutation on the chromosome causing the original phenotype. To overcome this complication, a random sample of positive- and negative-scoring recombinants can be tested using the screening assay to ensure that the two phenotypes correlate.

Recombinants for line 192-X have been generated using the OR strain as the appropriate reference strain for future single nucleotide polymorphism (SNP) mapping (Chen et al., 2008) (FIGURE 5.13). Line 192-X displayed abnormal locomotor activity aspects when compared to its control (FIGURE 5.9, FIGURE 5.11, AND TABLE 5-2). To date, 50 of the recombinants 192-X/OR recombinants have been phenotyped using the locomotor activity assay (TABLE 5-3). Each recombinant was given a score representing the degree of difference from the control in the phenotypes that 192-X was distinguished with. 67% of the tested recombinants displayed locomotor activity phenotypes similar to line 192-X. This number was higher than the 50% that would be expected based on random recombination. It is possible that this was due to screening a sample of 50 recombinants only, and that this percentage would reach the expected 50% as more recombinants are screened. The remainder of the recombination process will be carried out post-defense.
5.5 CHAPTER 5 FIGURES

**Figure 5.1** | Determination of EMS mutagenic efficiency by scoring for X-lethal mutations. **A.** Genetic crossing scheme employed. * indicates mutagenized chromosomes. Males were treated with 0, 5, 10, 15, 20, or 25mM of ems in 1% sucrose solution. Each group was then mated en masse to virgins carrying the homozygous viable X-chromosome balancer FM7. The adults were removed a few days later. All of the resulting F1 generation females were heterozygous carrying a mutagenized X chromosome and FM7. These F1 females were allowed to mate with their brothers and were transferred into vials individually. The absence of F2 males carrying a mutagenized X chromosome as opposed to FM7 indicates a lethal X chromosome mutation. **B.** Percentage of lines carrying viable versus lethal mutations after exposure to the different EMS concentrations. For each ems concentration, the percentage of lines carrying lethal and viable mutations were calculated. Unsuccessful vials were due to F1 females failing to produce progeny.
Figure 5.2 | Flowchart indicating the stages at which screening, re-testing, and different genetic crosses were conducted. * Indicates mutagenized chromosomes. // indicates “or”.

1. Establish Mutant Lines
   \[ \begin{align*}
   &+^*; +^*/+; +^*/+; +^*/+; +^*/Y; +^*/+ +^*/+; +^*/+; +^*/Y; +^*/+ +^*/+; +^*/+; +^*/Y; +^*/+ +^*/+; +^*/+ +^*/Y; +^*/+ +^*/+ +^*/Y; +^*/+ +^*/+ \end{align*} \]

2. Screen Males
   \[ \begin{align*}
   &+^*; +^*/+; +^*/+; +^*/+; +^*/Y; +^*/+ +^*/+; +^*/+; +^*/Y; +^*/+ +^*/+; +^*/+ +^*/Y; +^*/+ +^*/+ +^*/Y; +^*/+ +^*/+ +^*/Y; +^*/+ +^*/+ \end{align*} \]

3. Select Lines with interesting effects

4. Retest interesting lines.
   If phenotype confirmed

5. Isolate X chromosome
   \[ +^*; +; + +^*/Y; +^*/+; +^*/+ +^*/Y; +^*/+ +^*/+ +^*/Y; +^*/+ +^*/+ +^*/Y; +^*/+ +^*/+ \]

6. Test
   If phenotype persists

7. Establish 20 homozygous lines
   \[ \begin{align*}
   &+^*; +^*; +^*; +^*/Y; +^*/+; +^*/+ +^*/Y; +^*/+ +^*/+ +^*/Y; +^*/+ +^*/+ +^*/Y; +^*/+ +^*/+ \end{align*} \]

5. Isolate 2nd chromosome
   \[ +^*; +^*/+; + +^*/Y; +^*/+ +^*/+ +^*/Y; +^*/+ +^*/+ +^*/Y; +^*/+ +^*/+ \]

6. Test
   If phenotype persists

7. Establish 20 homozygous lines
   \[ \begin{align*}
   &+^*; +^*; +^*; +^*/Y; +^*/+; +^*/+ +^*/Y; +^*/+ +^*/+ +^*/Y; +^*/+ +^*/+ \end{align*} \]

5. Isolate 3rd chromosome
   \[ +^*; +^*/+; + +^*/Y; +^*/+ +^*/+ +^*/Y; +^*/+ +^*/+ +^*/Y; +^*/+ +^*/+ \]

6. Test
   If phenotype persists

7. Establish 20 homozygous lines
   \[ \begin{align*}
   &+^*; +^*; +^*; +^*/Y; +^*/+; +^*/+ +^*/Y; +^*/+ +^*/+ +^*/Y; +^*/+ +^*/+ \end{align*} \]
Mutagenized males hemizygous for mutations on the X chromosome, heterozygous for mutations on the 2nd and 3rd chromosomes, and that were selected for a confirmed effect in the social screen, were mated to virgin females. These females carried an attached X chromosome, and were heterozygous for 2nd and 3rd balancer chromosomes CyO and TM6B respectively. F1 males with 2nd and 3rd balancers were selected and mated to attached-X females with wild-type 2nd and 3rd chromosomes. F2 males carrying balancers for chromosomes 2 and 3 were selected to eliminate mutations previously residing on their autosomes. These F2 males were then mated to attached-X females with wild-type autosomes. Selection against balancers was the last step carried out to establish lines carrying mutations only on the X chromosome. The established lines were subsequently retested to investigate whether the phenotype detected in the parent line persists in the descendant lines.

Mutagenized males hemizygous for mutations on the X chromosome, heterozygous for mutations on the 2nd and 3rd chromosomes, and that were selected for a confirmed effect in the social screen, were mated to virgin females. These females carried an attached X chromosome, and were heterozygous for 2nd and 3rd balancer chromosomes CyO and TM6B respectively. F1 males with 2nd and 3rd balancers were selected and mated to attached-X females with wild-type 2nd and 3rd chromosomes. F2 males carrying balancers for chromosomes 2 and 3 were selected to eliminate mutations previously residing on their autosomes. These F2 males were then mated to attached-X females with wild-type autosomes. Selection against balancers was the last step carried out to establish lines carrying mutations only on the X chromosome. The established lines were subsequently retested to investigate whether the phenotype detected in the parent line persists in the descendant lines.

Mutagenized males hemizygous for mutations on the X chromosome, heterozygous for mutations on the 2nd and 3rd chromosomes, and that were selected for a confirmed effect in the social screen, were mated to virgin females. These females carried an attached X chromosome, and were heterozygous for 2nd and 3rd balancer chromosomes CyO and TM6B respectively. F1 males with 2nd and 3rd balancers were selected and mated to attached-X females with wild-type 2nd and 3rd chromosomes. F2 males carrying balancers for chromosomes 2 and 3 were selected to eliminate mutations previously residing on their autosomes. These F2 males were then mated to attached-X females with wild-type autosomes. Selection against balancers was the last step carried out to establish lines carrying mutations only on the X chromosome. The established lines were subsequently retested to investigate whether the phenotype detected in the parent line persists in the descendant lines.

Mutagenized males hemizygous for mutations on the X chromosome, heterozygous for mutations on the 2nd and 3rd chromosomes, and that were selected for a confirmed effect in the social screen, were mated to virgin females. These females carried an attached X chromosome, and were heterozygous for 2nd and 3rd balancer chromosomes CyO and TM6B respectively. F1 males with 2nd and 3rd balancers were selected and mated to attached-X females with wild-type 2nd and 3rd chromosomes. F2 males carrying balancers for chromosomes 2 and 3 were selected to eliminate mutations previously residing on their autosomes. These F2 males were then mated to attached-X females with wild-type autosomes. Selection against balancers was the last step carried out to establish lines carrying mutations only on the X chromosome. The established lines were subsequently retested to investigate whether the phenotype detected in the parent line persists in the descendant lines.

Mutagenized males hemizygous for mutations on the X chromosome, heterozygous for mutations on the 2nd and 3rd chromosomes, and that were selected for a confirmed effect in the social screen, were mated to virgin females. These females carried an attached X chromosome, and were heterozygous for 2nd and 3rd balancer chromosomes CyO and TM6B respectively. F1 males with 2nd and 3rd balancers were selected and mated to attached-X females with wild-type 2nd and 3rd chromosomes. F2 males carrying balancers for chromosomes 2 and 3 were selected to eliminate mutations previously residing on their autosomes. These F2 males were then mated to attached-X females with wild-type autosomes. Selection against balancers was the last step carried out to establish lines carrying mutations only on the X chromosome. The established lines were subsequently retested to investigate whether the phenotype detected in the parent line persists in the descendant lines.

Mutagenized males hemizygous for mutations on the X chromosome, heterozygous for mutations on the 2nd and 3rd chromosomes, and that were selected for a confirmed effect in the social screen, were mated to virgin females. These females carried an attached X chromosome, and were heterozygous for 2nd and 3rd balancer chromosomes CyO and TM6B respectively. F1 males with 2nd and 3rd balancers were selected and mated to attached-X females with wild-type 2nd and 3rd chromosomes. F2 males carrying balancers for chromosomes 2 and 3 were selected to eliminate mutations previously residing on their autosomes. These F2 males were then mated to attached-X females with wild-type autosomes. Selection against balancers was the last step carried out to establish lines carrying mutations only on the X chromosome. The established lines were subsequently retested to investigate whether the phenotype detected in the parent line persists in the descendant lines.

Mutagenized males hemizygous for mutations on the X chromosome, heterozygous for mutations on the 2nd and 3rd chromosomes, and that were selected for a confirmed effect in the social screen, were mated to virgin females. These females carried an attached X chromosome, and were heterozygous for 2nd and 3rd balancer chromosomes CyO and TM6B respectively. F1 males with 2nd and 3rd balancers were selected and mated to attached-X females with wild-type 2nd and 3rd chromosomes. F2 males carrying balancers for chromosomes 2 and 3 were selected to eliminate mutations previously residing on their autosomes. These F2 males were then mated to attached-X females with wild-type autosomes. Selection against balancers was the last step carried out to establish lines carrying mutations only on the X chromosome. The established lines were subsequently retested to investigate whether the phenotype detected in the parent line persists in the descendant lines.

Mutagenized males hemizygous for mutations on the X chromosome, heterozygous for mutations on the 2nd and 3rd chromosomes, and that were selected for a confirmed effect in the social screen, were mated to virgin females. These females carried an attached X chromosome, and were heterozygous for 2nd and 3rd balancer chromosomes CyO and TM6B respectively. F1 males with 2nd and 3rd balancers were selected and mated to attached-X females with wild-type 2nd and 3rd chromosomes. F2 males carrying balancers for chromosomes 2 and 3 were selected to eliminate mutations previously residing on their autosomes. These F2 males were then mated to attached-X females with wild-type autosomes. Selection against balancers was the last step carried out to establish lines carrying mutations only on the X chromosome. The established lines were subsequently retested to investigate whether the phenotype detected in the parent line persists in the descendant lines.

Mutagenized males hemizygous for mutations on the X chromosome, heterozygous for mutations on the 2nd and 3rd chromosomes, and that were selected for a confirmed effect in the social screen, were mated to virgin females. These females carried an attached X chromosome, and were heterozygous for 2nd and 3rd balancer chromosomes CyO and TM6B respectively. F1 males with 2nd and 3rd balancers were selected and mated to attached-X females with wild-type 2nd and 3rd chromosomes. F2 males carrying balancers for chromosomes 2 and 3 were selected to eliminate mutations previously residing on their autosomes. These F2 males were then mated to attached-X females with wild-type autosomes. Selection against balancers was the last step carried out to establish lines carrying mutations only on the X chromosome. The established lines were subsequently retested to investigate whether the phenotype detected in the parent line persists in the descendant lines.

Mutagenized males hemizygous for mutations on the X chromosome, heterozygous for mutations on the 2nd and 3rd chromosomes, and that were selected for a confirmed effect in the social screen, were mated to virgin females. These females carried an attached X chromosome, and were heterozygous for 2nd and 3rd balancer chromosomes CyO and TM6B respectively. F1 males with 2nd and 3rd balancers were selected and mated to attached-X females with wild-type 2nd and 3rd chromosomes. F2 males carrying balancers for chromosomes 2 and 3 were selected to eliminate mutations previously residing on their autosomes. These F2 males were then mated to attached-X females with wild-type autosomes. Selection against balancers was the last step carried out to establish lines carrying mutations only on the X chromosome. The established lines were subsequently retested to investigate whether the phenotype detected in the parent line persists in the descendant lines.
Figure 5.6 | Genetic crossing schemes employed to establish 20 individual lines homozygous for the autosomes from each putative 2nd and 3rd chromosome mutant lines. * Indicates chromosome of interest. // indicates “or”. Mutagenized males with either 2nd or 3rd chromosome mutations were not homozygous for the autosomes. This implies that some individuals within a line contain 2nd or 3rd chromosome mutations while others do not. In order to avoid losing mutations on the autosomes causing and effect in the social screen due to genetic drift, parent lines showing an effect in the social screen were crossed in order to extract around 20 individual chromosomes, used to establish 20 descendant lines from each parent line. Each of the descendant lines would be homozygous for either a mutant or wild-type autosome indicated by *. These descendant lines were generated in order to be tested individually in hopes of finding a homozygous line displaying an effect in the social screen as with the parent line. Without this step mapping would not be feasible since mapping requires homozygosity for the chromosome in question. A refers to crosses dealing with 2nd chromosome mutants, whereas B deals with 3rd chromosome mutations. Heat shocking was used to facilitate fly pushing whereby individuals with non-balancer and non-mutagenized autosomes were eliminated through the heat induced apoptotic gene hid.
Figure 5.7 | Comparison of some parent mutant lines with mutations on more than 1 chromosome to descendant lines with only one mutagenized chromosome. The influence of each line on the reporter’s PER-driven bioluminescence is compared. A RI of parent line 1414 is compared to the RI of its 1st, 2nd, and 3rd isolated chromosome descendants - lines 1414-X, 1414-2nd, and 1414-3rd respectively. B, line 86-2nd displays a phase shift that was not detected in the parent line 86. C, the mean counts/hr of parent line 192 is compared to all of its 1st, 2nd, and 3rd isolated chromosome descendant lines.
Figure 5.8 | Example figure illustrating the customized and automated analysis of locomotor activity. All plotted measures have been defined, generated, and discussed in Section 5.3.a. The circular plots displayed in N & O have been performed according to (Levine et al., 2002b). N. Dif represents phase difference in hours. Asterisks represent controls whereas circles represents the treatment line at hand. Numbers in parentheses indicate the number of replicate flies within a treatment. pD is a p-value for comparing differences in the distribution of within treatment phase values. pM is a p-value for comparing differences between average phase of treatments. O. phi represents average phase of a treatment. r represents average phase consistency of a treatment. p is a p-value resulting from the bivariate comparison of both groups taking phase and phase consistency into account.
Figure 5.9 | Analysis of locomotor activity of line 192-X compared to control in LD. All plotted measures have been defined, generated, and discussed in Section 5.3.a. The circular plots displayed in N & O have been performed according to (Levine et al., 2002b). N. Diff represents phase difference in hours. Asterisks represent controls whereas circles represents the treatment line at hand. Numbers in parentheses indicate the number of replicate flies within a treatment. pD is a p-value for comparing differences in the distribution of within treatment phase values. pM is a p-value for comparing differences between average phase of treatments. O. phi represents average phase of a treatment. r represents average phase consistency of a treatment. p is a p-value resulting from the bivariate comparison of both groups taking phase and phase consistency into account.
Figure 5.10 | Analysis of locomotor activity of line 1414-X compared to control in LD. All plotted measures have been defined, generated, and discussed in Section 5.3.a. The circular plots displayed in N & O have been performed according to (Levine et al., 2002b). N. Dif represents phase difference in hours. Asterisks represent controls whereas circles represents the treatment line at hand. Numbers in parentheses indicate the number of replicate flies within a treatment. pD is a $p$-value for comparing differences in the distribution of within treatment phase values. pM is a $p$-value for comparing differences between average phase of treatments. O. phi represents average phase of a treatment. r represents average phase consistency of a treatment. p is a $p$-value resulting from the bivariate comparison of both groups taking phase and phase consistency into account.
Figure 5.11 | Analysis of locomotor activity of line 192-X compared to control in DD. All plotted measures have been defined, generated, and discussed in Section 5.3.a. The circular plots displayed in N & O have been performed according to (Levine et al., 2002b). N. Dif represents phase difference in hours. Asterisks represent controls whereas circles represents the treatment line at hand. Numbers in parentheses indicate the number of replicate flies within a treatment. pD is a p-value for comparing differences in the distribution of within treatment phase values. pM is a p-value for comparing differences between average phase of treatments. O. phi represents average phase of a treatment. r represents average phase consistency of a treatment. p is a p-value resulting from the bivariate comparison of both groups taking phase and phase consistency into account.
Figure 5.12 | Analysis of locomotor activity of line 1414-X compared to control in DD. All plotted measures have been defined, generated, and discussed in Section 5.3.a. The circular plots displayed in N & O have been performed according to (Levine et al., 2002b). N, Dif represents phase difference in hours. Asterisks represent controls whereas circles represents the treatment line at hand. Numbers in parentheses indicate the number of replicate flies within a treatment. pD is a p-value for comparing differences in the distribution of within treatment phase values. pM is a p-value for comparing differences between average phase of treatments. O, phi represents average phase of a treatment. r represents average phase consistency of a treatment. p is a p-value resulting from the bivariate comparison of both groups taking phase and phase consistency into account.
Figure 5.14 | Genetic crossing scheme employed to insert BG-luc, tim-luc, or desat-luc into the background of candidate lines with mutation of interest mapping to the X chromosome. * indicates chromosome of interest. // indicates “or”. BG-luc, tim-luc, or desat-luc are all transgenes located on the 3rd chromosome. This crossing scheme was applied to EMS line 192-X and limbic line with a manipulation in the CG43243 locus.

Figure 5.13 | Genetic crossing scheme employed to generate recombinant lines for EMS line 192-X OR as a reference strain. * indicates chromosome of interest. // indicates “or”. Mutant 192-X males were crossed to OR females. Virgin females carrying recombinant X chromosome eggs from line 192-X and the OR strain were crossed to FM7 males. Single males carrying unique recombinant X chromosomes originating from females of the previous generation were used to establish individual unique lines by crossing to FM7 virgin females. Selection against X chromosome FM7 balancer was performed to establish lines from which males can be phenotyped and genotyped though SNP mapping (Chen et al., 2008). The locomotor activity phenotype of these lines is shown in Table 5-3.
Figure 5.15 | Spatial projection of pdf-positive neurons in line 192-X. Projections of pdf-positive neurons was visualized by pdf-GAL4 expression reported by UAS-mCD8::GFP (green) in dissected brains of A, wild-type adult males and B, 192-X adult males. In both lines, the s- and l-LN\textsubscript{v}s display normal projections. The s-LN\textsubscript{v}s project to the dorsal region of the brain whereas the l-LN\textsubscript{v}s project to the optic lobe (OL) as well as to the contralateral LN\textsubscript{v}s and OL through the posterior optic tract (POT). Both groups of LN\textsubscript{v}s are present in both brain hemispheres symmetrically.

Figure 5.16 | Analysis of courtship behaviour of line 192-X. Measures in which line 192-X significantly differs from its control are marked by asterisks. Males were monitored with females over a 10 minute time period. A, Courtship index is the percentage of the time invested in courtship behaviours including following, wing extension, and attempted copulation. Line 192-X spends significantly less time courting a wild-type female compared to its corresponding control, CSisoX (t-test p-value 0.00275). Wing extension index is the percentage of the time invested in wing extension and is not significantly different from the control. B, Courtship latency and mating latency represent the amount of time a male spends (seconds) before commencing courtship and successfully mating respectively. C, Attempted copulations measures the amount of times a male attempts copulation before successfully mating. D, Percent mated is number of males within a line that have successfully mated during the allocated time frame. Both line 192-X and its control display similar mating success rates.
## 5.6 Chapter 5 Tables

### Table 5-1 | Summary of lines with chromosome-mapped mutations and their corresponding effect on the reporter’s PER expression.

<table>
<thead>
<tr>
<th>Line #</th>
<th>Δ (Hrs.)</th>
<th>p-value</th>
<th>Line #</th>
<th>Δ (%)</th>
<th>p-value</th>
<th>Line #</th>
<th>Δ (%)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1036-X</td>
<td>1</td>
<td>0.002</td>
<td>298-X</td>
<td>-9.22</td>
<td>0.000</td>
<td>298-2nd</td>
<td>13</td>
<td>0.006</td>
</tr>
<tr>
<td>298-2nd *</td>
<td>1</td>
<td>0.001</td>
<td>1414-X</td>
<td>-9.16</td>
<td>0.004</td>
<td>1058-3rd</td>
<td>-11</td>
<td>0.027</td>
</tr>
<tr>
<td>86-2nd</td>
<td>1.46</td>
<td>0.001</td>
<td>192-X</td>
<td>-7.12</td>
<td>0.008</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>86-3rd</td>
<td>1.48</td>
<td>0.000</td>
<td>298-3rd **</td>
<td>-8.59</td>
<td>0.001</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>298-3rd **</td>
<td>1.1</td>
<td>0.002</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>192-3rd</td>
<td>1</td>
<td>0.001</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Line # is composed of the original mutant number and the chromosome to which the mutation has mapped. Δ represents difference from control. Negative Δ implies a decrease in the measure considered. p-value is that of t-test comparing each line to the control.

* Mutant line 298-2nd affects both the mean phase and RS of the accompanying reporter’s PER expression.

** Mutant line 298-3rd affects both the mean phase and mean counts/hr. of the accompanying reporter’s PER expression.

### Table 5-2 | Summary of lines with chromosome-mapped mutations and their corresponding locomotor activity phenotypes.

<table>
<thead>
<tr>
<th>Lights</th>
<th>Measure</th>
<th>1036_X</th>
<th>1414_X</th>
<th>192_X</th>
<th>298_2nd</th>
<th>86_2nd</th>
</tr>
</thead>
<tbody>
<tr>
<td>LD</td>
<td>Mean Day Counts</td>
<td>-35.47%</td>
<td>-55.17%</td>
<td>-41.54%</td>
<td>-29.34%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean Night Counts</td>
<td>-70.37%</td>
<td>-93.43%</td>
<td>-91.31%</td>
<td>-80.09%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean Night/Day Counts</td>
<td>-56.67%</td>
<td>-86.75%</td>
<td>-87.41%</td>
<td>-70.60%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean M peak Counts</td>
<td>-62.41%</td>
<td>-91.88%</td>
<td>-85.10%</td>
<td>-71.01%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean E peak Counts</td>
<td>-51.14%</td>
<td>-68.28%</td>
<td>-60.97%</td>
<td>-34.29%</td>
<td>-50.71%</td>
</tr>
<tr>
<td></td>
<td>Mean E/M Counts</td>
<td>619.27%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean Counts/24hrs</td>
<td>-55.22%</td>
<td>-76.83%</td>
<td>-69.70%</td>
<td>-29.26%</td>
<td>-58.06%</td>
</tr>
<tr>
<td></td>
<td>Mean Period (Autocorrelation)</td>
<td>-15.25%</td>
<td>-0.20%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean Rhythmicity Index</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean Rhythm Strength</td>
<td>-15.26%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean Phase</td>
<td>-16.45%</td>
<td>-17.39%</td>
<td>-21.16%</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean Phase Consistency</td>
<td>11.99%</td>
<td>13.54%</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| DD     | Mean Day Counts          | -58.7%  | -48.8%  |
|        | Mean Night Counts        | -63.4%  | -55.9%  | -59.9%  |
|        | Mean Night/Day Counts    |       | -34.1%  | -45.5%  |
|        | Mean M peak Counts       | -69.1%  | -58.6%  |
|        | Mean E peak Counts       | -57.4%  | -49.3%  | -48.1%  |
|        | Mean E/M Counts          |       | -36.4%  | -36.2%  |
|        | Mean Counts/24hrs        | -60.8%  | -52.0%  | -40.1%  |
|        | Mean Period (Autocorrelation) | -1.5%  | -2.1%  |
|        | Mean Rhythmicity Index   |       |
|        | Mean Rhythm Strength     | 29.8%   |
|        | Mean Phase               | -12.1%  | -18.8%  |
|        | Mean Phase Consistency   | 21.3%   |

Line # is composed of the original mutant number and the chromosome to which the mutation has mapped. Percentages represent difference from control. Negative numbers imply a decrease in the measure considered. Only measures with a corresponding t-test p-value less than 0.05 are shown.
Table 5-3 | Locomotor activity phenotypes of recombinants lines generated from EMS candidate line 192-X.

|----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|
For each of the considered measures, percent difference from the control is displayed. Negative numbers indicate percent decrease. Positive numbers indicate percent increase. 0% indicates no significant difference. The score is calculated by summing the number of measures highlighted in yellow that are significantly different from controls. A score of 3 and above suggests decreased. Positive numbers indicate percent increase.

* For each of the considered measures, percent difference from the control is displayed. Negative numbers indicate percent decrease. Positive numbers indicate percent increase. 0% indicates no significant difference. The score is calculated by summing the number of measures highlighted in yellow that are significantly different from controls. A score of 3 and above suggests decreased. Positive numbers indicate percent increase.

<table>
<thead>
<tr>
<th>RCSisoX_OR</th>
<th>0%</th>
<th>0%</th>
<th>0%</th>
<th>0%</th>
<th>0%</th>
<th>0%</th>
<th>0%</th>
<th>0%</th>
<th>0%</th>
<th>0%</th>
<th>0%</th>
<th>0%</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Significantly Different Lines</td>
<td>73%</td>
<td>11%</td>
<td>20%</td>
<td>20%</td>
<td>25%</td>
<td>16%</td>
<td>76%</td>
<td>75%</td>
<td>76%</td>
<td>20%</td>
<td>80%</td>
<td>73%</td>
</tr>
<tr>
<td># of Significantly Different Lines</td>
<td>40</td>
<td>6</td>
<td>11</td>
<td>11</td>
<td>14</td>
<td>9</td>
<td>42</td>
<td>41</td>
<td>42</td>
<td>11</td>
<td>44</td>
<td>40</td>
</tr>
<tr>
<td>Total # of Lines</td>
<td>55</td>
<td>55</td>
<td>55</td>
<td>55</td>
<td>55</td>
<td>55</td>
<td>55</td>
<td>55</td>
<td>55</td>
<td>55</td>
<td>55</td>
<td>55</td>
</tr>
</tbody>
</table>

* For each of the considered measures, percent difference from the control is displayed. Negative numbers indicate percent decrease. Positive numbers indicate percent increase. 0% indicates no significant difference. The score is calculated by summing the number of measures highlighted in yellow that are significantly different from controls. A score of 3 and above suggests decreased. Positive numbers indicate percent increase.

Table 5-4 | Hydrocarbon profile of line 192-X.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Class</th>
<th>CSisoX (Ctrl) (n=31)</th>
<th>EMS-192-X (n=31)</th>
<th>Comparison</th>
</tr>
</thead>
<tbody>
<tr>
<td>nC21</td>
<td>Alkane</td>
<td>21.8 ± (1.2)</td>
<td>19.9 ± (1.6)</td>
<td>-1.9 -9%</td>
</tr>
<tr>
<td>nC22</td>
<td>Alkane</td>
<td>45.5 ± (2.0)</td>
<td>28.7 ± (2.1)</td>
<td>-16.9 -37%</td>
</tr>
<tr>
<td>nC23</td>
<td>Alkane</td>
<td>243.6 ± (9.3)</td>
<td>157.2 ± (10.2)</td>
<td>-86.4 -35%</td>
</tr>
<tr>
<td>nC24</td>
<td>Alkane</td>
<td>14.5 ± (0.9)</td>
<td>8.1 ± (0.5)</td>
<td>-6.4 -44%</td>
</tr>
<tr>
<td>nC25</td>
<td>Alkane</td>
<td>41.3 ± (1.7)</td>
<td>33.0 ± (1.6)</td>
<td>-8.3 -20%</td>
</tr>
<tr>
<td>nC27</td>
<td>Alkane</td>
<td>22.7 ± (1.0)</td>
<td>20.0 ± (0.8)</td>
<td>-2.7 -12%</td>
</tr>
<tr>
<td>nC28</td>
<td>Alkane</td>
<td>2.3 ± (0.3)</td>
<td>2.0 ± (0.2)</td>
<td>-0.3 -14%</td>
</tr>
<tr>
<td>nC29</td>
<td>Alkane</td>
<td>6.8 ± (0.4)</td>
<td>8.9 ± (0.8)</td>
<td>2.2 32%</td>
</tr>
<tr>
<td>2MeC22</td>
<td>Methyl Alkane</td>
<td>4.2 ± (0.7)</td>
<td>4.4 ± (0.9)</td>
<td>0.2 5%</td>
</tr>
<tr>
<td>2MeC24</td>
<td>Methyl Alkane</td>
<td>41.8 ± (3.5)</td>
<td>21.3 ± (2.2)</td>
<td>-20.5 -49%</td>
</tr>
<tr>
<td>2MeC26</td>
<td>Methyl Alkane</td>
<td>59.3 ± (2.3)</td>
<td>46.8 ± (2.7)</td>
<td>-12.4 -21%</td>
</tr>
<tr>
<td>2MeC28</td>
<td>Methyl Alkane</td>
<td>53.2 ± (2.1)</td>
<td>65.6 ± (3.0)</td>
<td>12.5 23%</td>
</tr>
<tr>
<td>2MeC30</td>
<td>Methyl Alkane</td>
<td>64.5 ± (3.2)</td>
<td>64.7 ± (2.3)</td>
<td>0.1 0%</td>
</tr>
<tr>
<td>C23:1(5)</td>
<td>Monoene</td>
<td>32.0 ± (1.6)</td>
<td>29.9 ± (3.3)</td>
<td>-2.1 -7%</td>
</tr>
<tr>
<td>C23:1(7)</td>
<td>Monoene</td>
<td>758.2 ± (30.9)</td>
<td>603.3 ± (38.6)</td>
<td>-154.9 -20%</td>
</tr>
<tr>
<td>C23:1(9)</td>
<td>Monoene</td>
<td>58.2 ± (3.4)</td>
<td>58.9 ± (5.0)</td>
<td>0.7 1%</td>
</tr>
<tr>
<td>C24:1(5)</td>
<td>Monoene</td>
<td>15.9 ± (1.0)</td>
<td>8.2 ± (0.8)</td>
<td>-7.7 -49%</td>
</tr>
<tr>
<td>C24:1(9)+C24:1(7) Δ</td>
<td>Monoene</td>
<td>44.6 ± (2.9)</td>
<td>25.7 ± (2.1)</td>
<td>-18.8 -42%</td>
</tr>
<tr>
<td>C25:1(7)+C25:1(5) Δ</td>
<td>Monoene</td>
<td>371.4 ± (22.2)</td>
<td>189.5 ± (8.5)</td>
<td>-181.8 -49%</td>
</tr>
<tr>
<td>C25:1(9)</td>
<td>Monoene</td>
<td>85.2 ± (6.1)</td>
<td>39.1 ± (4.0)</td>
<td>-46.1 -54%</td>
</tr>
<tr>
<td>C27:1(7)</td>
<td>Monoene</td>
<td>14.5 ± (2.0)</td>
<td>8.4 ± (0.9)</td>
<td>-6.1 -42%</td>
</tr>
<tr>
<td>cVA</td>
<td>cVA</td>
<td>35.3 ± (3.3)</td>
<td>84.5 ± (12.9)</td>
<td>49.2 139%</td>
</tr>
<tr>
<td>nC18</td>
<td>Standard</td>
<td>466.9 ± (10.6)</td>
<td>475.3 ± (4.7)</td>
<td>8.5 2%</td>
</tr>
<tr>
<td>Alkanes</td>
<td>Total</td>
<td>398.7 ± (14.2)</td>
<td>277.7 ± (15.9)</td>
<td>-121.0 -30%</td>
</tr>
<tr>
<td>Methyl Alkanes</td>
<td>Total</td>
<td>222.9 ± (9.4)</td>
<td>202.7 ± (6.5)</td>
<td>-20.2 -9%</td>
</tr>
<tr>
<td>Monoenes</td>
<td>Total</td>
<td>1380.0 ± (60.2)</td>
<td>963.1 ± (52.5)</td>
<td>-416.9 -30%</td>
</tr>
<tr>
<td>cVA</td>
<td>Total</td>
<td>35.3 ± (3.3)</td>
<td>84.5 ± (12.9)</td>
<td>49.2 139%</td>
</tr>
<tr>
<td>Hydrocarbons</td>
<td>Total</td>
<td>2036.8 ± (80.0)</td>
<td>1528.2 ± (71.7)</td>
<td>-508.7 -25%</td>
</tr>
</tbody>
</table>

Δ represents the difference between the line in question and its respective control. Δ% indicates percent difference. Negative and positive differences indicate decreases and increases in the considered compounds respectively. The Δ column is color-scaled to help visualize the magnitude of differences. The grey color gradient is used for increases whereas the green color gradient is used for the decreases. The t-test p-value column marks significance where * implies <=0.05, ** implies <=0.005, and *** indicates <=0.0005. The different hydrocarbons are measured within one individual and are thus not independent measures. For this reason a false discovery rate adjustment was applied to the p-values before determining significance. Δ refers to compounds that do not separate well. This occurs for 2 reasons. First, both compounds have very similar retention times and are thus in extremely close-proximity. Second, the first compound is more abundant than the adjacent second compound. For these 2 reasons, these compounds fail to separate well and are thus summed together for better accuracy. Any differences that are observed in these sums are usually due to the more abundant compound. This has been confirmed empirically.
Chapter 6  REVERSE GENETIC APPROACH

6.1 SCREENING D. MELANOGASTER HOMOLOGUES OF MAMMALIAN LSAMP

One of the promising neurological models that attempt to explain psychosocial functioning is the limbic system model (Konner, 2010). In this model, certain regions of the mammalian brain referred to as the limbic lobe, constitute an essential anatomical component for the elaboration of social and adaptive behaviour (Heimer and Van Hoesen, 2006). It has also been proposed that this system underlies the occurrence of emotions such as fear of separation and fear of strangers (Heimer and Van Hoesen, 2006; Konner, 2010). Additionally, it is thought that interactions of hormones with this system account for the sex-specific differences in aggressive behaviour (Konner, 2010).

The cell and molecular dissection of the limbic system concept began after the discovery of limbic system-associated membrane protein (LSAMP) (Levitt, 1984). LSAMP codes for a cell adhesion molecule, whose expression is restricted mainly to the limbic structures (Pimenta et al., 1996). It plays a crucial role in the normal development of the limbic system circuitry (Pimenta et al., 1996).

LSAMP is conserved in a variety of species, including the structurally dissimilar avian brain (Yamamoto and Reiner, 2005). It was thus hypothesized that genetic homologues of LSAMP in the fly might have a conserved sociality-related function. Consequently, Ensembl was employed to retrieve LSAMP homologues in the fruitfly Figure 6.1. Homology in Ensembl is determined using gene trees that are based on the Gene Orthology/Paralogy prediction method pipeline (Vilella et al., 2009). Subsequent to identifying homologous loci, a search was conducted for fly lines with manipulations in some of these loci. Most identified lines were generated through a Gene Disruption Project involving a variety of transposons (Bellen et al., 2011; Thibault et al., 2004). These lines will hereon after be referred to as limbic mutants.

Several limbic mutant lines have been screened and analyzed using the same approach discussed in Section 4.4. The phenotypes of the examined lines have been documented in detail in Appendix A1.2.a. Of particular interest is Bloomington line 18681 with an insertion in locus CG42343. This line has not been previously studied and it therefore remains to be determined how this insertion affects gene expression, if at all. Regardless, this line was found to alter a reporter’s PER response to the presence of a companion. Among the differences observed, the most prominent were a decrease in the
reporter’s PER expression as well as a phase shift (Figure 6.2 B, C, D, E, J, N, & O). As a result, limbic mutant CG42343/18681 was chosen for further secondary characterization in this reverse genetic approach.

Other non-limbic candidates were also tested in the reverse approach. These are listed in detail in Appendix A 1.2.b.

6.2 Secondary Characterization of CG42343 Candidate

The same secondary characterization assays performed in the forward genetic approach were chosen to investigate further abnormalities associated with manipulating the CG42343 locus in limbic line 18681. This was done in an effort to understand the cells, tissues, and/or behaviours affected by the manipulation, and through which the influence on the reporter might be manifested. These include examining locomotor activity, clock gene expression, clock-related brain circuitry, phenomenal profiles, expression of the hydrocarbon synthesis enzyme DESAT, as well as courtship and mating behaviour. These investigations were chosen based on the same rational discussed in Section 5.3. Some of these secondary screens have already been completed. For others, some of the reagents have been generated and await further work post-defense or by other lab members.

6.2.a Locomotor Activity

The locomotor activity of limbic mutant 18681 with a manipulated CG42343 locus was examined. Although this line is characterized by decreased activity in both LD (Figure 6.3 A1, A2, C, D, E, & H) and DD (Figure 6.4 A1, A2, B, C, E, & H), a more striking phenotype was observed under DD conditions with respect to rhythmicity. In contrast to the control line, this limbic mutant became highly arrhythmic immediately upon switching light conditions to constant darkness. This was seen by examining the activity time series (Figure 6.4 A1 & A2), RI (Figure 6.4 J), and phase consistency (Figure 6.4 M & O). Although the bivariate analysis in Figure 6.4 O, cannot always confidently resolve whether a significant difference corresponds to mean phase and/or phase consistency, it is apparent that the latter is the source of significance. First, mean phase is not significantly different between the limbic mutant and the control when observed individually (Figure 6.4 L & N). Second, the data points corresponding to the limbic line are completely segregated from the control and are concentrated closer to the middle, indicating lower phase consistency (Figure 6.4 O). Such a strong segregation suggests that significance is reached due to differences in phase consistency.
6.2.b  CLOCK GENES EXPRESSION

In an effort to examine whether the limbic mutant displays normal clock gene expression, BG-luc and tim-luc were crossed into the background of line 18681. This manipulation permits examining PER and tim expression by monitoring bioluminescence. The crosses involved in creating these lines followed the same logic as shown in Figure 5.14. These lines have been generated and are now available in the Levine laboratory for future testing post-defense or by other lab members. One round of TC monitoring will provide sufficient insight into whether this candidate line exhibits normal PER and tim expression.

6.2.c  CLOCK-RELATED CIRCUITRY

Given the influence on PER expression the CG42343 candidate causes in the accompanying reporter, and the corresponding locomotor activity phenotypes, it was sound to question whether the number of clock-related brain neurons and their fasciculation is abnormal or not. As was reasoned in Section 5.3, the neurons chosen for this investigation were Pdf-positive neurons due to their crucial role in normal locomotor activity. Thus, Pdf-gal4 and UAS-gfp were crossed into the background of the line 18681 and the corresponding control. Brains were subsequently dissected and imaged using a GFP dissecting microscope or confocal microscope.

Preliminary investigations revealed that spatial projection of Pdf-gal4 expressing neurons reported by UAS-mCD8::GFP in dissected 18681 brains was similar to WT for both the s- and I-LNv (Data not shown). In both limbic mutant 18681 and WT controls, the s-LNv projected to the dorsal region of the brain. The I-LNv projected to the optic lobe as well as to the contralateral LNv and optic lobe through the posterior optic tract. Furthermore, the location and number of these neurons was also examined using Pdf-gal4 reported by UAS-mCD8::stinger expressed in the cell bodies of Pdf-positive neurons (Figure 6.5 A & B). This suggests that there does not exist visible developmental defects in clock-related circuitry of lines where the CG42343 locus has been manipulated. However, further clock-related neuronal groups need to be examined before a confident conclusion can be made in that regard.

6.2.d  HYDROCARBON PROFILES AND EXPRESSION OF THE HYDROCARBON SYNTHESIS ENZYME DESAT

As mentioned in the beginning of Section 5.3, it is plausible that the candidate line might have altered pheromonal profiles through which the influence on the reporter is communicated (Kent et al., 2008; Krupp et al., 2008; Levine et al., 2002a). Through a collaboration with a master’s student in the lab, the
hydrocarbons for line 18681 and the corresponding control were extracted and analyzed using gas chromatography. As expected, the hydrocarbon profile of 18681 males was significantly different from their corresponding control (TABLE 6-1). To begin with, the total amount of hydrocarbons found on the cuticle of these males was significantly higher than control males. The increase however was not an extremely prominent one (8%). This was the case since the hydrocarbon classes from which this total was calculated, deviate from the controls in opposing directions, minimizing the overall effect captured in the total. Furthermore, different hydrocarbons within each class also fluctuated in opposing directions. Although this was also observed in line 192-X, the inconsistency in directionalities was not as severe as observed in the limbic candidate. All alkanes except for nC22 were different from those of the control line. The shorter chain alkanes, nC21, nC23, nC24, and nC25, were significantly higher; whereas the longer chain alkanes, nC27, nC28, and nC29, were significantly less abundant. Methyl alkanes displayed a similar pattern. The shorter chain methyl alkanes, 2MeC22 and 2MeC24, displayed a significant increase. The longer chain methyl alkanes, 2MeC26, 2MeC28, and 2MeC30, exhibited a statistically significant decrease. Unlike alkanes and methyl alkanes, individual monoenes did not show a correlation between chain length and directionality of differences. Instead, both short and longer chain monoenes exhibited significant increases and decreases. For instance, C23:1(5) and C23:1(7) deviated from the control line by -32% and 36% respectively, while C25:1(9) and C27:1(7) differed by 83% and -53% respectively. cVA was not significantly different in this limbic line.

Since DESAT is a well-known enzyme involved in hydrocarbon synthesis, and whose expression has been shown to respond to the social environment (Kent et al., 2008), it was hypothesized that its expression in line 18681 might be different from that of the control. Thus the necessary crosses to insert desat-luc into the background of line 18681 and its control were carried out (FIGURE 5.14). This permits real time examination of desat-promoted luminescence in this line, providing in vivo data on desat expression. These lines have been generated and are now available in the Levine laboratory for future testing post-defense or by other lab members.

6.2.e COURTSHIP AND MATING BEHAVIOUR

Since courtship and mating behaviour have been found highly responsive to the social environment both in a group and pair setting (Fujii and Amrein, 2010; Fujii et al., 2007; Krupp et al., 2008), it was also interesting to examine whether the limbic line with manipulated CG42343 locus displayed normal courtship and mating behaviour. Males were monitored with females over a 10 minute time period
using LIFESONG software (Bernstein et al., 1992). Courtship analysis included monitoring measures such as courtship index, wing extension index, courtship latency, mating latency, attempted copulations, and percent mated defined in Section 9.5.

The limbic mutant line demonstrated severely impaired courtship behaviour (Figure 6.6). Limbic mutant males were not impaired in the time taken to recognize the presence of a female as captured by the courtship latency measure (Figure 6.6 B). Their invested efforts in attempted copulations were also comparable to controls (Figure 6.6 C). However, they invested significantly less time courting than their control counterparts (Figure 6.6 A), and within the time allocated for courtship, they displayed much less wing extension. Most importantly, none of the limbic mutant males mated within the allocated time frame (Figure 6.6 D).
Identification of *D. melanogaster* homologues of mammalian LSAMP using Ensembl. Homology in Ensembl is determined using gene trees that are based on the Gene Orthology/Paralogy prediction method pipeline (Vilella et al., 2009).
Figure 6.2 | Analysis of PER-driven bioluminescence of reporter in the presence of limbic mutant line compared to a reporter co-housed with a wild-type conspecific. All plotted measures have been defined and discussed in Section 4.3. The generation and analysis of all included measures have been performed as described in 0. The circular plots displayed in L to O have been performed according to (Levine et al., 2002b). L & N. Dif represents phase difference in hours. Asterisks represent controls whereas circles represents the treatment line at hand. Numbers in parentheses indicate the number of replicate flies within a treatment. pD is a p-value for comparing differences in the distribution of within treatment phase values. pM is a p-value for comparing differences between average phase of treatments. M & O, phi represents average phase of a treatment. r represents average phase consistency of a treatment. p is a p-value resulting from the bivariate comparison of both groups taking phase and phase consistency into account.
Figure 6.3 | Analysis of locomotor activity of limbic mutant line compared to control in LD. All plotted measures have been defined, generated, and discussed in Section 5.3.a. The circular plots displayed in N & O have been performed according to (Levine et al., 2002b). N. Dif represents phase difference in hours. Asterisks represent controls whereas circles represents the treatment line at hand. Numbers in parentheses indicate the number of replicate flies within a treatment. pD is a p-value for comparing differences in the distribution of within treatment phase values. pM is a p-value for comparing differences between average phase of treatments. O. phi represents average phase of a treatment. r represents average phase consistency of a treatment. p is a p-value resulting from the bivariate comparison of both groups taking phase and phase consistency into account.
Figure 6.4 | Analysis of locomotor activity of limbic mutant line compared to control in DD. All plotted measures have been defined, generated, and discussed in Section 5.3.a. The circular plots displayed in N & O have been performed according to (Levine et al., 2002b). N. Dif represents phase difference in hours. Asterisks represent controls whereas circles represents the treatment line at hand. Numbers in parentheses indicate the number of replicate flies within a treatment. pD is a p-value for comparing differences in the distribution of within treatment phase values. pM is a p-value for comparing differences between average phase of treatments. phi represents average phase of a treatment. r represents average phase consistency of a treatment. p is a p-value resulting from the bivariate comparison of both groups taking phase and phase consistency into account.
Figure 6.5 | Count of *pdf*-positive neurons in limbic mutant CG42343 (18681). The cell bodies of *pdf*-positive neurons were visualized by *pdf-GAL4* expression reported by *UAS-stinger* (green) in dissected brains of **A**, wild-type adult males and **B**, 18681 adult males. In both lines, the amount of s- and l-LNvS seem normal (4 each). Both groups of LNvS are present in both brain hemispheres symmetrically.
Figure 6.6 | Analysis of courtship behaviour of limbic line with manipulated CG42343 locus. Measures in which limbic mutant significantly differs from its control are marked by asterisks. Males were monitored with females over a 10 minute time period. A, Courtship index is the percentage of the time invested in courtship behaviours including following, wing extension, and attempted copulation. Limbic mutant (n=10) spent significantly less time courting a wild-type female compared to its corresponding control, w1118 (n=10) (p-value 0.02695). Wing extension index is the percentage of the time invested in wing extension and was also significantly different from the control (p-value 0.02695). B, Courtship latency and mating latency represent the amount of time a male spends (seconds) before commencing courtship and successfully mating respectively. Since none of the limbic mutant males mated, their mating latency was set to 600 (latest monitoring time to facilitate testing significance (p-value 0.02695). C, Attempted copulations measures the amount of times a male attempts copulation before successfully mating. D, Percent mated is number of males within a line that have successfully mated during the allocated time frame. None of the limbic mutant males mated within the allocated time frame (chi-squared test 0.0098).
### 6.4 Chapter 6 Tables

<table>
<thead>
<tr>
<th>Compound</th>
<th>Class</th>
<th>Avg ± (SEM) (ng)</th>
<th>Δ (ng)</th>
<th>Δ %</th>
<th>t-test p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>nC21</td>
<td>Alkane</td>
<td>23.2 ± (0.9)</td>
<td>2.3</td>
<td>10%</td>
<td>*</td>
</tr>
<tr>
<td>nC22</td>
<td>Alkane</td>
<td>32.7 ± (1.6)</td>
<td>1.8</td>
<td>5%</td>
<td></td>
</tr>
<tr>
<td>nC23</td>
<td>Alkane</td>
<td>152.3 ± (5.1)</td>
<td>58.2</td>
<td>38%</td>
<td>***</td>
</tr>
<tr>
<td>nC24</td>
<td>Alkane</td>
<td>9.7 ± (0.5)</td>
<td>1.0</td>
<td>11%</td>
<td>*</td>
</tr>
<tr>
<td>nC25</td>
<td>Alkane</td>
<td>28.0 ± (1.0)</td>
<td>15.6</td>
<td>56%</td>
<td>***</td>
</tr>
<tr>
<td>nC27</td>
<td>Alkane</td>
<td>23.1 ± (1.0)</td>
<td>-2.5</td>
<td>-11%</td>
<td>*</td>
</tr>
<tr>
<td>nC28</td>
<td>Alkane</td>
<td>2.9 ± (0.2)</td>
<td>-1.6</td>
<td>-57%</td>
<td>***</td>
</tr>
<tr>
<td>nC29</td>
<td>Alkane</td>
<td>8.8 ± (0.7)</td>
<td>-4.2</td>
<td>-48%</td>
<td>***</td>
</tr>
<tr>
<td>2MeC22</td>
<td>Methyl Alkane</td>
<td>0.2 ± (0.1)</td>
<td>5.1</td>
<td>265%</td>
<td>***</td>
</tr>
<tr>
<td>2MeC24</td>
<td>Methyl Alkane</td>
<td>11.8 ± (0.8)</td>
<td>34.5</td>
<td>291%</td>
<td>***</td>
</tr>
<tr>
<td>2MeC26</td>
<td>Methyl Alkane</td>
<td>84.5 ± (4.2)</td>
<td>-8.7</td>
<td>-10%</td>
<td>*</td>
</tr>
<tr>
<td>2MeC28</td>
<td>Methyl Alkane</td>
<td>89.1 ± (2.4)</td>
<td>-35.5</td>
<td>-40%</td>
<td>***</td>
</tr>
<tr>
<td>2MeC30</td>
<td>Methyl Alkane</td>
<td>72.1 ± (2.4)</td>
<td>-7.6</td>
<td>-11%</td>
<td>**</td>
</tr>
<tr>
<td>C23:1(5)</td>
<td>Monoene</td>
<td>39.3 ± (3.0)</td>
<td>-12.6</td>
<td>-32%</td>
<td>***</td>
</tr>
<tr>
<td>C23:1(7)</td>
<td>Monoene</td>
<td>563.8 ± (20.1)</td>
<td>203.1</td>
<td>36%</td>
<td>***</td>
</tr>
<tr>
<td>C23:1(9)</td>
<td>Monoene</td>
<td>53.5 ± (2.7)</td>
<td>-1.8</td>
<td>-3%</td>
<td></td>
</tr>
<tr>
<td>C24:1(5)</td>
<td>Monoene</td>
<td>13.7 ± (0.6)</td>
<td>-3.8</td>
<td>-28%</td>
<td>***</td>
</tr>
<tr>
<td>C24:1(9)+C24:1(7)</td>
<td>Monoene</td>
<td>39.0 ± (1.8)</td>
<td>-5.8</td>
<td>-15%</td>
<td>*</td>
</tr>
<tr>
<td>C25:1(7)+C25:1(5)</td>
<td>Monoene</td>
<td>297.5 ± (11.6)</td>
<td>-95.2</td>
<td>-32%</td>
<td>***</td>
</tr>
<tr>
<td>C25:1(9)</td>
<td>Monoene</td>
<td>28.4 ± (1.6)</td>
<td>23.6</td>
<td>83%</td>
<td>***</td>
</tr>
<tr>
<td>C27:1(7)</td>
<td>Monoene</td>
<td>9.0 ± (0.8)</td>
<td>-4.7</td>
<td>-53%</td>
<td>***</td>
</tr>
<tr>
<td>cVA</td>
<td>cVA</td>
<td>150.8 ± (22.5)</td>
<td>-21.4</td>
<td>-14%</td>
<td></td>
</tr>
<tr>
<td>nC18</td>
<td>Standard</td>
<td>467.4 ± (4.7)</td>
<td>3.6</td>
<td>1%</td>
<td></td>
</tr>
<tr>
<td>Alkanes</td>
<td>Total</td>
<td>280.5 ± (9.7)</td>
<td>70.8</td>
<td>25%</td>
<td>***</td>
</tr>
<tr>
<td>Methyl Alkanes</td>
<td>Total</td>
<td>257.6 ± (7.3)</td>
<td>-12.0</td>
<td>-5%</td>
<td></td>
</tr>
<tr>
<td>Monoenes</td>
<td>Total</td>
<td>1044.2 ± (38.2)</td>
<td>102.8</td>
<td>10%</td>
<td>*</td>
</tr>
<tr>
<td>cVA</td>
<td>Total</td>
<td>150.8 ± (22.5)</td>
<td>-21.4</td>
<td>-14%</td>
<td></td>
</tr>
<tr>
<td>Hydrocarbons</td>
<td>Total</td>
<td>1733.2 ± (52.2)</td>
<td>140.2</td>
<td>8%</td>
<td>*</td>
</tr>
</tbody>
</table>

Δ represents the difference between the line in question and its respective control. Δ% indicates percent difference. Negative and positive differences indicate decreases and increases in the considered compounds respectively. The Δ column is color-scaled to help visualize the magnitude of differences. The grey color gradient is used for increases whereas the green color gradient is used for the decreases. The t-test p-value column marks significance where * implies <=0.05, ** implies <=0.005, and *** indicates <=0.0005. The different hydrocarbons are measured within one individual and are thus not independent measures. For this reason a false discovery rate adjustment was applied to the p-values before determining significance. \( \delta \) refers to compounds that do not separate well. This occurs for 2 reasons. First, both compounds have very similar retention times and are thus in extremely close-proximity. Second, the first compound is more abundant than the adjacent second compound. For these 2 reasons, these compounds fail to separate well and are thus summed together for better accuracy. Any differences that are observed in these sums are usually due to the more abundant compound. This has been confirmed empirically.
Chapter 7  Discussion

7.1  Social Clock Plasticity Likely Functions Within The Confiness Of Photoperiod Input

Using a novel assay consisting of a dyadic social context, PER expression of a focal individual was monitored in the presence of an interacting stimulus fly. Deviations in the circadian signal of the focal fly were used to identify novel genes in the stimulus fly that underlie social interactions. Using a forward genetic approach, a total of 2552 EMS lines were generated (Figure 5.3) and screened (Figure 5.2 Steps 1 & 2) in the presence of a reporter. Fourteen lines caused deviated PER responses in the co-housed reporter, compared to a reporter’s PER response in the presence of a control. A series of crosses were conducted to isolate the X, 2<sup>nd</sup>, and 3<sup>rd</sup> chromosome from each of the 14 potential mutant lines (Figure 5.2 Step 5), in an effort to identify the chromosome on which the mutation of interest resides. Thirty six descendant lines were generated (14 lines x 3 chromosomes - 6 failed crosses) and re-screened, of which 10 lines were chosen for further investigation. The phenotype of the reporter in the presence of each of the 10 putative mutants, was grouped into 3 categories defined in Section 4.3 (Table 5-1). Six lines produced a phase shift in the reporter’s rhythmic PER expression compared to a reporter in the presence of a control. Four lines caused a change in the reporter’s overall PER expression levels, and 2 lines caused a change in the reporter’s rhythmicity index (defined in Section 4.3). Out of these, 2 lines produced changes in 2 measures simultaneously. Two types of expression changes were rarely observed, those in phase consistency and period, also defined in Section 4.3. Phase consistency captures the consistency in time of day at which a reporter’s PER expression peaks over several days. Period represents the time it takes to complete one full cycle of a repeated expression pattern. That none of the EMS mutant lines examined induced a change in these measures on their accompanying reporter, might be due to the LD settings in which the screen was conducted. It is possible that while social information is able to modulate an individual’s clock status, it can only do so within strict limitations given that light is a much stronger Zeitgeber. For instance, while phase shifts can take place, they do so in a consistent manner from day to day, maintaining overall consistency in peak timing and spacing. Thus it appears that the strict boundaries imposed by light apply to key facets of rhythmic expression such as phase and period. Other aspects seem to be more responsive to social input, and are thus inherently more variable. These include patterns between peak intervals and/or peak amplitude. This explains the occurrence of mutants inducing changes in the reporter’s RI. This measure quantifies the degree of pattern reproducibility across repeated cycles, and can thus capture
changes in peak amplitude and pattern variability between peaks. As a result, although light imposes strict control over the degree of phase variability, more flexibility appears to exist when dealing with peak amplitude and expression patterns spanning between peak intervals. This ‘cap’ on the type and extent of influence the social environment can inflict on PER expression seems biologically sound. It is rational for individuals to carry out activities such as foraging in synchrony with major abiotic factors like photoperiod. For instance, predator avoidance and foraging success might be more effective during the daytime. Subsequently, individuals would then fine tune the timing of such activities within the day according to social conditions. For example, engaging in increased foraging during the day might be necessary in the presence of a larger group of conspecifics due to competition, while protection from predators might be more effective. Following this line of thought, it is thus not surprising that social clock plasticity might be tightly regulated by abiotic factors, and fine-tuned by social input. This accounts for not uncovering mutations that change a companion’s PER expression period and phase consistency in an LD cycle. Finally, understanding how such social signals integrate along with photoperiod information into an individual’s clock mechanism also requires testing reporters in the presence of each of the mutant lines in DD. These studies are ongoing but are not a part of this thesis.

7.2 Multiple Loci, Likely Operating In Different Pathways Including the Clock Mechanism, Might Be Involved In Inflicting Social Influence On Conspecifics.

None of the EMS mutants discussed have been fully mapped, making it premature to discuss any of the findings in the context of specific loci. This is the case since failure to map mutations is a common occurrence, because a phenotype could be the result of more than one mutation within a chromosome. Having said that, working under the possibility that at least one of the uncovered mutations might be mapped successfully, in combination with results from some candidate mutants whose loci are known, few hypotheses can be made.

H 1 - Multiple loci bring about social influence. When considering the genomic location of the 10 candidate EMS mutations, 2 lines harbored mutations mapping to the 2nd chromosome, 4 lines to the 3rd, and 4 lines mapping to the X chromosome (Table 5-1). Furthermore, the candidate limbic line that was identified through the reverse genetic approach, harbored an insertion in the CG42343 locus located on the X chromosome. Other candidates with mutations in known loci, listed in Appendix A.1.2, also caused an effect in the social screen. This implies that multiple loci on different chromosomes
within the fly genome could mediate the influence of the social environment on its members. This is not surprising since most molecular, cellular, and/or physiological pathways naturally require more than one player. Most proteins carrying out a function interact with target molecules, and most neurons generating a signal transduce the message to a downstream target. This however, does not necessitate that all affected genes operate within the same pathway, as will be discussed shortly.

**H 2 - Some mutations might affect the clock mechanism of mutants.** In an effort to investigate the cells, tissues, and/or behaviours affected by the mutations, and through which the influence on the reporter might be manifested, several secondary characterization assays were conducted. This was based on the expectation that some abnormalities might be associated with these mutations. The logic supporting this expectation is that these individuals must be in a dissimilar physiological state compared to a WT individual. Otherwise, it would be difficult to envision an explanation as to why their social message to the reporter was different from that of controls.

Since the screening assay selected for mutations in an individual that alter PER expression in a companion reporter, it was reasonable to question the clock status of the mutants themselves. The plausibility of this question arose from previous findings documenting the role of clocks in inflicting social influence on a companion, as discussed in the reviews presented Chapter 1 AND Chapter 2. For instance, clock mutants have been shown to exert an influence on the locomotor activity and clock gene expression of co-housed WT conspecifics (Krupp et al., 2008; Levine et al., 2002a). More specifically, per mutants decreased the amplitude of DD host per expression in the head and oenocytes. One finding challenges the role of the mutants’ clock mechanism in inducing a social effect on the companion reporter in this screen. When per mutants were tested in the screening assay, the reporter’s PER response was similar to that in the presence of a WT individual (Figure A.32). This finding however does not completely refute the role of clocks detected by Krupp et al. (2008). It is possible that the extra copies of per expressed in the reporter as a result of the BG-luc transgene, increase the threshold of the social input required to detect changes in PER expression. Regardless, the net message is that the role of clocks in this novel screening assay cannot be completely disregarded (Chapter 2).

Since locomotor activity is a clock-controlled behaviour, monitoring locomotor activity was one of the assays employed to probe for clock-related defects in the putative mutants. The locomotor activity phenotypes of some candidate lines are summarized in Table 5-2 and are documented in detail in Appendix II. Indeed, most of the tested candidate lines displayed locomotor activity patterns that were
significantly different from controls in several aspects. This indicates that, if successfully mapped, some mutations might reside in loci operating in or affecting the clock system. It is also worth noting that when considering the collective locomotor activity phenotypes of the candidate lines, most differences occurred during LD settings as opposed to DD. This is not surprising since the screen was conducted in LD.

**H 3 - Multiple pathways for the generation of social signals might exist.** Although the possibility that at least some of the selected candidate mutant lines harbor mutations in clock genes or in genes affecting the circadian system is valid, it is not the only plausible possibility. It is reasonable to expect that more than one mechanism exists for inflicting social influences on an individual. One piece of evidence supporting this possibility is that mutants for the *Cyp6a20* gene induce a change in the way a reporter responds to companionship. More specifically, reporters in the presence of the *Cyp6a20* mutants exhibit an increase in PER expression compared to a reporter in the presence of a WT male (Figure A.31). The *Cyp6a20* effect complements previous data, since *Cyp6a20* is well known to mediate the effect of social experience on aggressive behaviour, and is thought to be involved in pheromone sensitivity (Dierick, 2008; Wang et al., 2008). This strongly suggests that more than one mechanism exists for mediating social influence on an individual. It is rational to conclude so, since one individual can produce more than one message that get subsequently integrated into the recipient’s physiological state. For instance, it has already been established that individuals communicate gender and species identity through pheromonal tags (Billeter et al., 2009), and that this information modulates more than one behavioural response such as mating and/or aggression (Billeter et al., 2012b; Fernandez et al., 2010; Garbaczewska et al., 2013; Krupp et al., 2008; Wang et al., 2011). Other pathways might include those involved in hydrocarbon synthesis or transport, as evidenced by the hydrocarbon abnormalities observed in line 192-X and the limbic candidate. Consequently, it is likely that at least some of these mutations might reside in loci that could function within different pathways, apart from circadian, to convey a variety of messages that eventually influence the clock status of a companion.

**H 4 - Some mutations might reside in pleiotropic loci.** Whether the mutations in question reside in clock genes or not, two further potential likelihoods can be considered. While belonging to the clock mechanism or to an independent non-clock function, these mutations might reside in pleiotropic loci, where the influence on the reporter and on the mutants’ own locomotor activity arise through independent pathways (Figure 7.1 A.1, ii & A.2, 1i). Conversely, it is also possible that these mutations alter the mutants’ locomotor activity patterns, which in turn influences the reporter’s clock mechanism.
Another unconsidered scenario is that a mutation in question operates in a function that is upstream of, but that eventually feeds in into, the clock mechanism (Figure 7.1 A.3). For instance a mutation could map to a gene involved in circadian photoreception such as in the Hofbauer-Buchner eyelet (Veleri et al., 2007).

### 7.3 Secondary Characterization of Line 192-X

Several EMS mutants were shown to cause an effect on the reporter’s PER expression in the social screen (Table 5-1). Of those, line 192-X showed a similar effect to line 1414-X. Furthermore, the locomotor activity phenotypes of both lines were similar (Table 5-2). Analogous to the reduction in PER expression they inflicted on the reporter in the screening assay, day and nighttime activity of these lines were significantly reduced in LD and DD (Table 5-2, Figure 5.9, Figure 5.10, Figure 5.11, & Figure 5.12 B, C, D, E, &H). Furthermore, both lines displayed a phase shift in locomotor activity in LD and not DD (Table 5-2, Figure 5.9, Figure 5.10, Figure 5.11, & Figure 5.12 L, N, & O), as well as an increase in phase consistency in LD (Table 5-2, Figure 5.9, Figure 5.10 M). The only difference between the two lines occurred in DD where line 192-X’s phase consistency was higher (Table 5-2, Figure 5.11, & Figure 5.12 M). Despite the latter discrepancy between the two, it is possible that the corresponding mutations map to the same gene. Unfortunately, as a result of the mutations’ location on the X chromosome, it is impossible to carry out complementation tests in males, to confirm this possibility. Regardless, this increases the likelihood that such a mutation is ‘mappable’, since it is unlikely for more than one mutation to occur in the exact same loci in more than one line. As a result these two lines were of special interest, and line 192-X was chosen for further characterization and mapping since it was healthier than 1414-X.

Further characterization of EMS line 192-X was carried out. Given the PER expression influence this line caused in the accompanying reporter, along with the observed locomotor activity phenotype, it was sound to question whether the number of clock-related brain neurons and their fasciculation is abnormal. Pdf-positive neurons were chosen due to their crucial role in normal locomotor activity (Hardin, 1994). Spatial projection of Pdf-gal4 expressing neurons reported by UAS-mCD8::GFP in 192-X brains was similar to WT for both the s- and l-LNs (Figure 5.15). This suggests the lack of developmental defects in the fasciculation in PDF-related circuitry of this line. However, further clock-related neuronal groups need to be examined before a confident conclusion can be made in that regard. It would also be interesting to examine other non-clock neuronal groups within the brain. This
is necessary since as mentioned previously, the influence inflicted by this line on the accompanying reporter does not necessarily have to be mediated through the clock mechanism.

The pheromonal profile of line 192-X was also examined, since it is possible that the influence on the reporter is communicated through olfaction, as was found in previous studies (Fujii and Amrein, 2010; Levine et al., 2002a). Indeed, the hydrocarbon profile of line 192-X males was significantly different from that of the corresponding control (Table 5-4). An overall reduction of 25% was observed when considering total amount of hydrocarbons. This decrease cannot be attributed to a possible reduction in the overall size of males. This is because hydrocarbon classes and hydrocarbons within each of the classes fluctuated with varying magnitudes and directions. For instance, although alkanes exhibited a 30% reduction in total amounts, nC29 was 32% higher than in the corresponding control line. Similar to alkanes, monoenes were also reduced. Methyl alkanes exhibited a unique pattern of differences from the control line. The two 2nd shortest methyl alkanes, 2MeC24 and 2MeC26 were reduced, whereas the 2nd longest methyl alkane 2MeC28 was more abundant. The compound that displayed the most striking difference from the control line was cVA. Line 192-X expressed more than double the amount of cVA found in the control line. The striking magnitude of this increase was not observed in any of the other compound. Since cuticular hydrocarbons are an established mechanism of communication between individuals (Billeter et al., 2009), they constitute an extremely likely mechanism with which the social influence on the reporter is communicated. For instance, C23:1(7) or 7-T is repulsive to other males and may prevent male–male sexual interactions (Lacaille et al., 2007). 7-T was significantly reduced in 192-X mutants which might induce reporters to court 192-X males. Since the occurrence of courtship is known to alter a male’s clock status as discussed in Chapter 2, 7-T might be one signal altering the response of a reporter to the presence of a mutant companion. Furthermore, cVA has been shown to stimulate male–male aggression (Wang and Anderson, 2010). Since 192-X males display an immense increase in cVA, the reporter’s clock status might have been altered to allow for aggressive behaviour. One experiment that could further confirm or refute such hypotheses is testing anosmic reporters in the presence 192-X males. If confirmed, these findings would be in agreement with previous data, whereby olfactory cues have been found to mediate the influence of the social environment on clock-controlled behaviours such as locomotor activity (Fujii and Amrein, 2010; Fujii et al., 2007; Levine et al., 2002a). Additionally, they would agree with the model suggesting that the clock mechanism might act as a hub for the integration of social input and subsequent modulation of behaviours (Section 2.5).
The increase in cVA observed in line 192-X compared to controls might explain this line’s courtship and mating phenotype. 192-X males displayed similar courtship characteristics to controls in all respects except for courtship index, which was significantly less (Figure 5.16). Since percent mated and mating latency were not significantly different from controls, this implied that 192-X males mated within the normal time frame, yet simply spent less time courting. Furthermore, levels of wing extension and attempted copulation carried out within the time invested in courtship were maintained at WT ratios as well. Such a courtship profile indicates that although this line courts less on average, it is still able to successfully mate within the WT time frame. Yet, instead of successfully mating faster, these males simply spend less effort courting to achieve the same results a WT male achieves. This could imply that males of this mutant line are more attractive than their respective controls, making females more receptive to their efforts. Increased levels of cVA might underlie such possible increase in female receptivity.

7.4 SECONDARY CHARACTERIZATION OF LIMBIC CANDIDATE

Bloomington line 18681 with an insertion in locus CG42343, was found to alter a reporter’s PER response to the presence of a companion (Figure 6.2 B, C, D, E, J, N, & O). Further characterization of this line was carried out. Its locomotor activity exhibited a decrease in both LD (Figure 6.3 A1, A2, C, D, E, & H) and DD (Figure 6.4 A1, A2, B, C, E, & H). Furthermore, a more striking phenotype was observed under DD conditions with respect to rhythmicity. In contrast to the control line, this limbic mutant became highly arrhythmic immediately upon switching light conditions to constant darkness. This was seen by examining the activity time series (Figure 6.4 A1 & A2), RI (Figure 6.4 J), and phase consistency (Figure 6.4 M & O). It is again not surprising that a transgenic line influencing PER expression in the reporter displays abnormal locomotor activity itself. This is because as discussed in Chapter 2, the time-keeping system of both interacter and interactee seem to be involved, or as previously worded ‘communicate’, in the mediation of social influence on activity.

Similar to the secondary screening of 192-X, clock-related neurons were assayed, using Pdf-gal4. Preliminary results indicated that there was no difference in the spatial projection of Pdf-gal4 expressing neurons reported by UAS-mCD8::GFP compared to WT for both the s- and l-LNv's (Data not shown). Furthermore, no difference was observed in the number of Pdf-gal4 expressing neurons reported by UAS-mCD8::stinger compared to WT for both the s- and l-LNv's (Figure 6.5 A & B). It may still
be premature to form a conclusion in that regard, hence further clock-related neuronal groups need to be examined.

The hydrocarbon profile of transgenic limbic males was significantly different from controls (Table 6-1). The hydrocarbons of this line significantly differed from controls in opposing directions that were not class-specific. Although this was also observed in line 192-X, the inconsistency in directionalities was not as grave as that observed in the limbic candidate. Almost all alkanes were different from controls. The shorter chain alkanes were significantly higher, whereas the longer chain alkanes were significantly less abundant. Methyl alkanes displayed a similar pattern. This indicates that an abnormality in hydrocarbon lengthening or excessive degradation of longer chain compounds might exist. Unlike alkanes and methyl alkanes, individual monoenes did not show a correlation between chain length and directionality of differences. Furthermore, cVA was not significantly different in this line. As is the case with line 192-X, it is possible that the altered pheromonal profile in this line underlies changes in the accompanying reporter’s clock status. Again, such a possibility would be in agreement with previous data, whereby olfactory cues have been found to mediate the influence of the social environment on clock-controlled behaviours such as locomotor activity (Fujii and Amrein, 2010; Fujii et al., 2007; Levine et al., 2002a).

It is difficult to attribute this line’s challenges in successful mating (Figure 6.6) to the abnormalities in pheromonal profiles. As captured by the courtship latency measure, limbic mutant males were not impaired in the time taken to recognize the presence of a female (Figure 6.6 B). Their invested efforts in attempted copulations were also comparable to controls (Figure 6.6 C). However, they invested significantly less time courting than their control counterparts (Figure 6.6 A), and within the time allocated for courtship, they displayed much less wing extension. Most importantly, none of the limbic mutant males mated within the allocated time frame (Figure 6.6 D). As a result, since courtship efforts were significantly diminished, this suggests that the underlying problem is not a reduction in female receptivity due to the altered pheromonal profile. Understanding the site of action of CG42343’s product will be crucial in jump-starting our understanding of its function.

7.5 Potential Mechanisms Through Which Line 192-X and the Limbic Candidate Influence a Companion’s Clock

What resonates from findings related to line 192-X and the limbic candidate is that the potentially affected loci alter locomotor activity, hydrocarbon profiles, as well as courtship behaviour, along with
influencing the reporter. Consequently these functions can be included into the network of mechanistic possibilities when planning future investigations (Figure 7.1). While belonging to the clock mechanism or to an independent non-clock function, these mutations might reside in pleiotropic loci, where the influence on the reporter, on the mutants’ own locomotor activity, and hydrocarbon profile arise through independent pathways (Figure 7.1 B.1.iii & Figure 7.1 B.2.iii). Conversely, it is also possible that these mutations alter the mutants’ locomotor activity patterns or hydrocarbon profiles, which in turn influence the reporter’s clock mechanism (Figure 7.1 B.1.i & B.1.ii respectively and Figure 7.1 B.2.i & B.2.ii respectively). Another scenario is that the mutations in question operate in a function that is upstream of but that eventually feeds in into the clock mechanism (Figure 7.1 B.3.i, B.3.ii, & B.3.iii). Needless to say, mapping the EMS mutation and further characterization of the limbic line will provide conclusive answers to these questions.
7.6 Chapter 7 Figures

Figure 7.1 | Framework depicting possible pathways through which a gene in a candidate line inflicts a social influence on a companion reporter. Pathways within the light grey boxes represent mechanisms within the mutant line. Pathways within the dark grey boxes represent mechanisms within the reporter. The “→” sign represents “influences”. Letters labelling the arrows were chosen to better represent the action at hand (TL=effect on reporter occurs through locomotor activity, TH=effect on reporter occurs through hydrocarbons, L=influence on locomotor activity, H=influence on hydrocarbons, I=direct independent effect on reporter. Effects that are not mediated through the circadian system are indicated by prime (’).

A. Framework depicting possible pathways of influencing the reporter without considering hydrocarbons. A.1.i, mutant’s clock gene alters locomotor activity (path L) which in turn affects the reporter (path TL). A.1.ii, mutant’s clock gene is pleiotropic. It alters locomotor activity (path L) and influences the reporter (path L’) through independent pathways. A.2.i, mutant’s gene X alters locomotor activity (path L’), which in turn affects reporter (path TL). A.2.ii, mutant’s gene X is pleiotropic. It alters locomotor activity (path L’) and influences the reporter (path L”) through independent pathways.

A.3, mutant’s gene X feeds into clock mechanism (path X) which in turn influences the reporter through any of earlier described pathways. Briefly, mutant’s gene X feeds into clock mechanism (path X). A.3.i The altered clock system in turn regulates locomotor activity (path L) which in turn affects the reporter (path TL). A.3.ii, Alternatively, the altered clock system regulates locomotor activity (path L) and influences the reporter (path L’ through independent pathways. B. Framework depicting possible pathways while incorporating the mutation’s effect on hydrocarbons. This model accounts for mutations that influence both locomotor activity and hydrocarbon expression in the mutant, as well as PER expression in a companion (as was seen in line 192-X and the limbic candidate line). The same logic is used as in A to depict the different possibilities. For instance, if the identified gene is a clock gene, then it could operate by B.1.i, regulating locomotor activity (path L) which in turn affects the reporter (path TL), while influencing hydrocarbons independently (path H). B.1.ii, Alternatively, the clock gene could affect hydrocarbons (path H), which in turn affect the reporter (path TH), while influencing locomotor activity independently (path L). B.1.ii Another possibility is that this clock gene affects locomotor activity (path L), hydrocarbon profiles (path H), and the reporter (path L) through independent pathways. The same logic applies to B.2, and B.3.
Chapter 8  CONCLUSION

Several studies have highlighted the involvement of clocks in mediating the effect of social experience on numerous D. melanogaster behaviours (Fujii et al., 2007; Krupp et al., 2008; Levine et al., 2002a). More specifically, clocks on both the emitter and receiver end, seem to be integral in mediating social interactions; whereby both systems ‘communicate’ to generate a coordinated response (CHAPTER 2). It is also plausible that circadian clocks act as a hub for the integration of social signals and the appropriate modulation of downstream behaviours, be it courtship, aggression, or other unidentified interactions.

The work presented in this thesis aimed at introducing new pillars in the framework established in CHAPTER 2. More specifically, biochemical and cellular pathways within an emitter involved in triggering social influence on a recipient’s clock were investigated. Although such an approach might appear to naively linearize inter-individual interactions, this was solely done to facilitate the investigations at hand. In reality, it is clear that group members cannot be strictly labelled as emitters or receivers, because these functions are linked and are dynamic within and between individuals in a social setting. It is believed that such interactions are more complex and involve recurrent feedback between individuals, to fine-tune group members’ physiological and behavioural statuses.

A forward and reverse genetic approach were undertaken to identify genes/mutations within an individual that influence PER expression in a companion. Through an EMS mutagenesis, 10 candidate mutant lines were identified. Through the reverse approach, one line with a manipulation in a locus homologous to the LSAMP was selected. Other candidates were also identified, but focus was placed on the one limbic line for logistical reasons. Collectively, the aforementioned candidates induced phase shifts, changes in expression levels, and/or changes in RI of PER expression in a co-housed companion. Changes in phase consistency and period were not observed. Since the screen was conducted in LD, it is likely that while social information is able to modulate an individual’s clock status, it can only do so within the confines of photoperiod input, given that light is a much stronger Zeitgeber.

Of the 10 candidate EMS mutant lines, 2 lines harbored mutations mapping to the 2nd chromosome, 4 lines to the 3rd, and 4 lines mapping to the X chromosome. Furthermore, the candidate limbic line that was identified through the reverse genetic approach, harbored an insertion in the CG42343 locus located on the X chromosome. This implies that multiple loci on different chromosomes within the fly
genome might be involved in mediating the influence of the social environment on its members. This however, does not necessitate that all affected genes operate within the same pathway. Since locomotor activity is a clock-controlled behaviour, it was one of the assays employed to probe for clock-related defects within the candidates. Indeed, most of the tested candidate lines displayed locomotor activity patterns that were significantly different from controls. This hinted to the possibility that at least some of these mutations reside in clock genes or affect the clock system. However, keeping in mind that other tested candidates harboring mutations in non-clock loci such as Cyp6a20 also influenced the reporter, it is reasonable that more than one mechanism exists for inflicting social influences on an individual.

The hydrocarbon profiles and courtship behaviours of lines 192-X and the limbic candidate line were significantly altered. This suggests that the social influence of these lines on the accompanying reporters might be mediated by olfaction. For instance, 7-T was significantly reduced in 192-X mutants. Thus it is possible that this decrease might induce reporters to court 192-X males (Lacaille et al., 2007), which in turn alters the reporter’s time-keeping system (Fujii et al., 2007). Furthermore, cVA has been shown to stimulate male–male aggression (Wang and Anderson, 2010). Since 192-X males display an increase in cVA, the reporter’s clock status might have been altered to allow for aggressive behaviour.

This thesis presents new findings that introduce new guidelines to our understanding of the mechanisms underlying social interactions. Evidence is provided regarding the existence of multiple loci within an individual, likely operating in multiple pathways, and that are involved in exerting social influence on conspecifics. One such pathway is likely the circadian clock system. It has also been established that such putative loci also modulate locomotor activity, hydrocarbon production, as well as courtship behaviour. While it is challenging to map out the involvement of these functions in the generation of a social signal, a scheme of possibilities was synthesized in order to direct future investigations (Figure 7.1). Within this scheme, the potential pleiotropic characteristic of the mutations in questions is considered. Finally, an improved analysis package was generated to fully automate and significantly increase throughput of future investigations. This thesis therefore represents a new methodology that has already begun to dissect the complex interactions that exist within the social life of Drosophila melanogaster.
Chapter 9  METHODS

A substantial portion of the methods employed have been presented in the main body of the thesis. This was necessary to permit the discussion of protocols that were specifically developed for this research, and were hence an important portion of my progress. For instance, methods pertaining to the analysis of bioluminescence have been included in Section 4.4. This was necessary since a significant portion of the employed MATLAB scripts was newly developed throughout my research, and required thorough justification. Another example includes methods pertaining to the chemical mutagenesis which have been included in Section 5.1. This was done to permit the justification of experimental details such as EMS dosage. The EMS dosage chosen was as a result of the lethality experiment conducted and presented in Figure 5.1. The methods listed below constitute well established protocols and have been briefly described.

9.1  FLY REARING

All fly strains were raised on a nutrient medium containing the following ingredients: agar, glucose, sucrose, yeast, cornmeal, wheat germ, soya flour, molasses, propionic acid, and Tegosept. Experimental stocks were maintained in 177.4 ml square bottom, polypropylene bottles containing 8 ml of food in a 12 hr. LD cycle at 25°C.

9.2  TC BIOLUMINESCENCE MONITORING

Bioluminescence monitoring was carried out as described in (Brandes et al., 1996). Briefly, 0-2 day old males were collected using CO2 anesthesia post eclosion. These males were reared in same sex groups of 20 individuals, and aged for 3-4 days in an LD cycle, in vials (25 x 85 mm) containing 1 ml of food. On the day of the experiment, males were briefly anesthetized on ice and placed in every well of a 96-well microtiter plate from Perkin Elmer, containing nutrient media supplemented with luciferin. Luciferin was obtained from Biosynth Europe Ltd. In addition to luciferin, the nutrient media contained bactoagar and sucrose. The plates were then placed in a Perkin Elmer TopCount multi-plate scintillation counter, programmed to measure bioluminescence counts automatically once per hour per well for a period of 5 days. The 1st 24 hours of data are not included in subsequent analysis.

9.3  LOCOMOTOR ACTIVITY TESTING

0-2 day old males were collected using CO2 anesthesia post eclosion. These males were reared in same sex groups of 20 individuals, and aged for 3-4 days in an LD cycle, in vials (25 x 85 mm) containing 1 ml of food. On the day of the experiment, males were individually placed in Trikinetics activity tubes via an aspirator. The tubes contained minimal nutrient media (agar and sucrose) at one end and a cotton
plug at the other end. The tubes were placed in Trikinetics activity monitors. Locomotor activity was subsequently recorded for at least 7 days in LD and 7 days in DD.

9.4 Cuticular Hydrocarbon Analysis

0-1 day old males were collected using CO2 anesthesia post eclosion. These males were reared in same sex groups of 20 individuals, and aged for 4-5 days in an LD cycle, in vials (25 x 85 mm) containing 1 ml of food. Hydrocarbons were extracted as performed in (Billeter et al., 2009). Briefly, flies were anesthetized with ether and placed into individual glass microvials (Agilent) containing 50 ml of hexane supplemented with 10 ng/ml of the two internal standards, nC18 and nC26. Vials were subsequently agitated for 2 minutes. Hydrocarbons were processed using gas chromatography (Varian CP3800) with a flame ionization detector. Star Integrator software (Varian) was used for compound quantification based on peak areas.

9.5 Courtship Behaviour Analysis

0-1 day old males were collected using CO2 anesthesia post eclosion. These males were reared in same sex groups of 20 individuals, and aged for 4-5 days in an LD cycle, in vials (25 x 85 mm) containing 1 ml of food. Courtship analysis was carried out as was performed in (Billeter et al., 2009). Males and virgin females were aspirated into cylindrical Plexiglas chambers (10mm diameter 35mm depth) and video monitored for 10 minutes. Courtship analysis included monitoring measures such as courtship index, wing extension index, courtship latency, mating latency, attempted copulations, and percent mated. Courtship index was calculated as the percentage of the time a male invested in courtship behaviours including following, wing extension, and attempted copulation. If a male mated within the monitored 10 minute period, then total time was set to time-to-mating in the calculation of this index. Otherwise, total time used in the calculation was set to 10 minutes (600 seconds). Wing extension index was calculated as the percentage of the time invested in wing extension. Courtship latency and mating latency represented the amount of time a male spent in seconds before commencing courtship and successfully mating respectively. Attempted copulations measured the amount of times a male attempted copulation before successfully mating. Percent mated is number of males within a line that have successfully mated during the allocated time frame.
REFERENCES


Appendix I  PHENOTYPES OF CANDIDATE LINES IN SOCIAL SCREEN

A.I.1  FORWARD GENETIC APPROACH CANDIDATES

In total, 10 mutant EMS lines were chosen for further investigation. These are listed in Table 5-1, and PER expression of their accompanying reporters is shown in the figures below. For each line, analysis of reporter PER expression is shown using flies and experiments as replicates.
Figure A. 1 | Analysis of PER-driven bioluminescence of a reporter with a 86-2nd male compared to a reporter co-housed with a wild-type conspecific using flies as replicates. All plotted measures have been defined and discussed in Section 4.3. The generation and analysis of all included measures have been performed as described in Section 4.4. The circular plots displayed in L to O have been performed according to (Levine et al., 2002b). L & N. Dif represents phase difference in hours. Asterisks represent controls whereas circles represents the treatment line at hand. Numbers in parentheses indicate the number of replicate flies within a treatment. pD is a \( p \)-value for comparing differences in the distribution of within treatment phase values. pM is a \( p \)-value for comparing differences between average phase of treatments. M & O, \( \phi \) represents average phase of a treatment. \( r \) represents average phase consistency of a treatment. p is a \( p \)-value resulting from the bivariate comparison of both groups taking phase and phase consistency into account.
Figure A. 2 | Analysis of PER-driven bioluminescence of a reporter with a 86-2nd male compared to a reporter co-housed with a wild-type conspecific using individual experiments as replicates. All plotted measures have been defined and discussed in Section 4.3. The generation and analysis of all included measures have been performed as described in Section 4.4.
Figure A. 3 | Analysis of PER-driven bioluminescence of a reporter with an 86-3rd male compared to a reporter co-housed with a wild-type conspecific using flies as replicates. All plotted measures have been defined and discussed in Section 4.3. The generation and analysis of all included measures have been performed as described in Section 4.4. The circular plots displayed in L to O have been performed according to Levine et al., 2002b. L & N, Dif represents phase difference in hours. Asterisks represent controls whereas circles represents the treatment line at hand. Numbers in parentheses indicate the number of replicate flies within a treatment. pD is a p-value for comparing differences in the distribution of within treatment phase values. pM is a p-value for comparing differences between average phase of treatments. M & O, phi represents average phase of a treatment. r represents average phase consistency of a treatment. p is a p-value resulting from the bivariate comparison of both groups taking phase and phase consistency into account.
Figure A. 4 | Analysis of PER-driven bioluminescence of a reporter with a 86-3rd male compared to a reporter co-housed with a wild-type conspecific using individual experiments as replicates. All plotted measures have been defined and discussed in Section 4.3. The generation and analysis of all included measures have been performed as described in Section 4.4.
Figure A. 5 | Analysis of PER-driven bioluminescence of a reporter with a 192-3rd male compared to a reporter co-housed with a wild-type conspecific using flies as replicates. All plotted measures have been defined and discussed in Section 4.3. The generation and analysis of all included measures have been performed as described in Section 4.4. The circular plots displayed in L to O have been performed according to (Levine et al., 2002b). L & N. Dif represents phase difference in hours. Asterisks represent controls whereas circles represents the treatment line at hand. Numbers in parentheses indicate the number of replicate flies within a treatment. pD is a p-value for comparing differences in the distribution of within treatment phase values. pM is a p-value for comparing differences between average phase of treatments. M & O, phi represents average phase of a treatment. r represents average phase consistency of a treatment. p is a p-value resulting from the bivariate comparison of both groups taking phase and phase consistency into account.
Figure A. 6 | Analysis of PER-driven bioluminescence of a reporter with a 192-3rd male compared to a reporter co-housed with a wild-type conspecific using individual experiments as replicates. All plotted measures have been defined and discussed in Section 4.3. The generation and analysis of all included measures have been performed as described in Section 4.4.
Figure A. 7 | Analysis of PER-driven bioluminescence of a reporter with a 192-X male compared to a reporter co-housed with a wild-type conspecific using flies as replicates. All plotted measures have been defined and discussed in Section 4.3. The generation and analysis of all included measures have been performed as described in Section 4.4. The circular plots displayed in L to O have been performed according to Levine et al., 2002b. L & N. Dif represents phase difference in hours. Asterisks represent controls whereas circles represents the treatment line at hand. Numbers in parentheses indicate the number of replicate flies within a treatment. pD is a \( p \)-value for comparing differences in the distribution of within treatment phase values. pM is a \( p \)-value for comparing differences between average phase of treatments. M & O. phi represents average phase of a treatment. r represents average phase consistency of a treatment. p is a \( p \)-value resulting from the bivariate comparison of both groups taking phase and phase consistency into account.

A

B

C

D

E

F

G

H

I

J

K

L

M

N

O
Figure A. 8 | Analysis of PER-driven bioluminescence of a reporter with a 192-X male compared to a reporter co-housed with a wild-type conspecific using individual experiments as replicates. All plotted measures have been defined and discussed in Section 4.3. The generation and analysis of all included measures have been performed as described in Section 4.4.
Figure A. 9 | Analysis of PER-driven bioluminescence of a reporter with a 298-2nd allele compared to a reporter co-housed with a wild-type conspecific using flies as replicates. All plotted measures have been defined and discussed in Section 4.3. The generation and analysis of all included measures have been performed as described in Section 4.4. The circular plots displayed in L to O have been performed according to (Levine et al., 2002b). L & N. Dif represents phase difference in hours. Asterisks represent controls whereas circles represent the treatment line at hand. Numbers in parentheses indicate the number of replicate flies within a treatment. pD is a p-value for comparing differences in the distribution of within treatment phase values. pM is a p-value for comparing differences between average phase of treatments. M & O, phi represents average phase of a treatment. r represents average phase consistency of a treatment. p is a p-value resulting from the bivariate comparison of both groups taking phase and phase consistency into account.
Figure A. 10 | Analysis of PER-driven bioluminescence of a reporter with a 298-29\textsuperscript{nd} male compared to a reporter co-housed with a wild-type conspecific using individual experiments as replicates. All plotted measures have been defined and discussed in Section 4.3. The generation and analysis of all included measures have been performed as described in Section 4.4.
Figure A.11 | Analysis of PER-driven bioluminescence of a reporter with a 298-3rd male compared to a reporter co-housed with a wild-type conspecific using flies as replicates. All plotted measures have been defined and discussed in Section 4.3. The generation and analysis of all included measures have been performed as described in Section 4.4. The circular plots displayed in L to O have been performed according to (Levine et al., 2002b). L & N. Dif represents phase difference in hours. Asterisks represent controls whereas circles represent the treatment line at hand. Numbers in parentheses indicate the number of replicate flies within a treatment. pD is a p-value for comparing differences in the distribution of within treatment phase values. pM is a p-value for comparing differences between average phase of treatments. M & O. phi represents average phase of a treatment. r represents average phase consistency of a treatment. p is a p-value resulting from the bivariate comparison of both groups taking phase and phase consistency into account.
Figure A. 12 | Analysis of PER-driven bioluminescence of a reporter with a 298-3rd male compared to a reporter co-housed with a wild-type conspecific using individual experiments as replicates. All plotted measures have been defined and discussed in Section 4.3. The generation and analysis of all included measures have been performed as described in Section 4.4.
Figure A. 13 | Analysis of PER-driven bioluminescence of a reporter with a 298-X male compared to a reporter co-housed with a wild-type conspecific using flies as replicates. All plotted measures have been defined and discussed in Section 4.3. The generation and analysis of all included measures have been performed as described in Section 4.4. The circular plots displayed in L to O have been performed according to (Levine et al., 2002b). L & N. Dif represents phase difference in hours. Asterisks represent controls whereas circles represents the treatment line at hand. Numbers in parentheses indicate the number of replicate flies within a treatment. pD is a p-value for comparing differences in the distribution of within treatment phase values. pM is a p-value for comparing differences between average phase of treatments. M & O, phi represents average phase of a treatment. r represents average phase consistency of a treatment. p is a p-value resulting from the bivariate comparison of both groups taking phase and phase consistency into account.
Figure A. 14 | Analysis of PER-driven bioluminescence of a reporter with a 298-X male compared to a reporter co-housed with a wild-type conspecific using individual experiments as replicates. All plotted measures have been defined and discussed in Section 4.3. The generation and analysis of all included measures have been performed as described in Section 4.4.
Figure A. 15 | Analysis of PER-driven bioluminescence of a reporter with a 1036-X male compared to a reporter co-housed with a wild-type conspecific using flies as replicates. All plotted measures have been defined and discussed in Section 4.3. The generation and analysis of all included measures have been performed as described in Section 4.4. The circular plots displayed in L to O have been performed according to (Levine et al., 2002b). L & N. Dif represents phase difference in hours. Asterisks represent controls whereas circles represents the treatment line at hand. Numbers in parentheses indicate the number of replicate flies within a treatment. pD is a p-value for comparing differences in the distribution of within treatment phase values. pM is a p-value for comparing differences between average phase of treatments. M & O. phi represents average phase of a treatment. r represents average phase consistency of a treatment. p is a p-value resulting from the bivariate comparison of both groups taking phase and phase consistency into account.
Figure A. 16 | Analysis of PER-driven bioluminescence of a reporter with a 1036-X male compared to a reporter co-housed with a wild-type conspecific using individual experiments as replicates. All plotted measures have been defined and discussed in Section 4.3. The generation and analysis of all included measures have been performed as described in Section 4.4.
Figure A. 17 | Analysis of PER-driven bioluminescence of a reporter with a 1058-3rd male compared to a reporter co-housed with a wild-type conspecific using flies as replicates. All plotted measures have been defined and discussed in Section 4.3. The generation and analysis of all included measures have been performed as described in Section 4.4. The circular plots displayed in L to O have been performed according to (Levine et al., 2002b). L & N. Dif represents phase difference in hours. Asterisks represent controls whereas circles represents the treatment line at hand. Numbers in parentheses indicate the number of replicate flies within a treatment. pD is a p-value for comparing differences in the distribution of within treatment phase values. pM is a p-value for comparing differences between average phase of treatments. M & O. phi represents average phase of a treatment. r represents average phase consistency of a treatment. p is a p-value resulting from the bivariate comparison of both groups taking phase and phase consistency into account.
Figure A. 18 | Analysis of PER-driven bioluminescence of a reporter with a 1058-3rd male compared to a reporter co-housed with a wild-type conspecific using individual experiments as replicates. All plotted measures have been defined and discussed in Section 4.3. The generation and analysis of all included measures have been performed as described in Section 4.4.
Figure A. 19 | Analysis of PER-driven bioluminescence of a reporter with a 1414-X male compared to a reporter co-housed with a wild-type conspecific using flies as replicates. All plotted measures have been defined and discussed in Section 4.3. The generation and analysis of all included measures have been performed as described in Section 4.4. The circular plots displayed in L to O have been performed according to (Levine et al., 2002b). L & N. Dif represents phase difference in hours. Asterisks represent controls whereas circles represents the treatment line at hand. Numbers in parentheses indicate the number of replicate flies within a treatment. pD is a p-value for comparing differences in the distribution of within treatment phase values. pM is a p-value for comparing differences between average phase of treatments. M & O. phi represents average phase of a treatment. r represents average phase consistency of a treatment. p is a p-value resulting from the bivariate comparison of both groups taking phase and phase consistency into account.
Figure A. 20 | Analysis of PER-driven bioluminescence of a reporter with a 1414-X male compared to a reporter co-housed with a wild-type conspecific using individual experiments as replicates. All plotted measures have been defined and discussed in Section 4.3. The generation and analysis of all included measures have been performed as described in Section 4.4.
A.1.2 REVERSE GENETIC APPROACH CANDIDATES

A.1.2.a LIMBIC CANDIDATES

Several lines with manipulations in loci homologous to mammalian LSAMP were screened. These are listed in Table A.1. Lines causing a significant change in the reporter’s PER expression compared to a reporter housed with a control, are shown below.

<table>
<thead>
<tr>
<th>Bloomington Line Number</th>
<th>Locus</th>
</tr>
</thead>
<tbody>
<tr>
<td>18681</td>
<td>Dmel\CG42343</td>
</tr>
<tr>
<td>22944</td>
<td>Dmel\CG42343</td>
</tr>
<tr>
<td>23035</td>
<td>Dmel\CG32791</td>
</tr>
<tr>
<td>23182</td>
<td>Dmel\CG31708</td>
</tr>
<tr>
<td>23405</td>
<td>Dmel\CG11320</td>
</tr>
<tr>
<td>23625</td>
<td>Dmel\CG42343</td>
</tr>
<tr>
<td>24693</td>
<td>Dmel\CG14521</td>
</tr>
<tr>
<td>27790</td>
<td>Dmel\CG31646</td>
</tr>
<tr>
<td>28654</td>
<td>Dmel\CG31646</td>
</tr>
<tr>
<td>32808</td>
<td>Dmel\CG42343</td>
</tr>
<tr>
<td>34278</td>
<td>Dmel\CG14521</td>
</tr>
<tr>
<td>34458</td>
<td>Dmel\CG32791</td>
</tr>
<tr>
<td>35926</td>
<td>Dmel\CG31646</td>
</tr>
<tr>
<td>35928</td>
<td>Dmel\CG14521</td>
</tr>
<tr>
<td>36970</td>
<td>Dmel\CG31708</td>
</tr>
</tbody>
</table>
Figure A. 21 | Analysis of PER-driven bioluminescence of a reporter with a 18681 male compared to a reporter co-housed with a wild-type conspecific using flies as replicates. All plotted measures have been defined and discussed in Section 4.3. The generation and analysis of all included measures have been performed as described in Section 4.4. The circular plots displayed in L to O have been performed according to Levine et al. (2002b). L & N. Dif represents phase difference in hours. Asterisks represent controls whereas circles represent the treatment line at hand. Numbers in parentheses indicate the number of replicate flies within a treatment. pD is a p-value for comparing differences in the distribution of within treatment phase values. pM is a p-value for comparing differences between average phase of treatments. M & O. phi represents average phase of a treatment. r represents average phase consistency of a treatment. p is a p-value resulting from the bivariate comparison of both groups taking phase and phase consistency into account.
Figure A. 22 | Analysis of PER-driven bioluminescence of a reporter with a 22944 male compared to a reporter co-housed with a wild-type conspecific using flies as replicates. All plotted measures have been defined and discussed in Section 4.3. The generation and analysis of all included measures have been performed as described in Section 4.4. The circular plots displayed in L to O have been performed according to (Levine et al., 2002b). L & N. Dif represents phase difference in hours. Asterisks represent controls whereas circles represents the treatment line at hand. Numbers in parentheses indicate the number of replicate flies within a treatment. pD is a $p$-value for comparing differences in the distribution of within treatment phase values. pM is a $p$-value for comparing differences between average phase of treatments. M & O. phi represents average phase of a treatment. r represents average phase consistency of a treatment. p is a $p$-value resulting from the bivariate comparison of both groups taking phase and phase consistency into account.
Figure A. 23 | Analysis of PER-driven bioluminescence of a reporter with a 23035 male compared to a reporter co-housed with a wild-type conspecific using flies as replicates. All plotted measures have been defined and discussed in Section 4.3. The generation and analysis of all included measures have been performed as described in Section 4.4. The circular plots displayed in L to O have been performed according to (Levine et al., 2002b). L & N. Dif represents phase difference in hours. Asterisks represent controls whereas circles represents the treatment line at hand. Numbers in parentheses indicate the number of replicate flies within a treatment. pD is a p-value for comparing differences in the distribution of within treatment phase values. pM is a p-value for comparing differences between average phase of treatments. M & O. phi represents average phase of a treatment. r represents average phase consistency of a treatment. p is a p-value resulting from the bivariate comparison of both groups taking phase and phase consistency into account.
Figure A. 24 | Analysis of PER-driven bioluminescence of a reporter with a 23182 male compared to a reporter co-housed with a wild-type conspecific using flies as replicates. All plotted measures have been defined and discussed in Section 4.3. The generation and analysis of all included measures have been performed as described in Section 4.4. The circular plots displayed in L to O have been performed according to (Levine et al., 2002b). L & N. Dif represents phase difference in hours. Asterisks represent controls whereas circles represents the treatment line at hand. Numbers in parentheses indicate the number of replicate flies within a treatment. pD is a p-value for comparing differences in the distribution of within treatment phase values. pM is a p-value for comparing differences between average phase of treatments. M & O. phi represents average phase of a treatment. r represents average phase consistency of a treatment. p is a p-value resulting from the bivariate comparison of both groups taking phase and phase consistency into account.
Figure A. 25 | Analysis of PER-driven bioluminescence of a reporter with a 23625 male compared to a reporter co-housed with a wild-type conspecific using flies as replicates. All plotted measures have been defined and discussed in Section 4.3. The generation and analysis of all included measures have been performed as described in Section 4.4. The circular plots displayed in L to O have been performed according to (Levine et al., 2002b). L & N. Dif represents phase difference in hours. Asterisks represent controls whereas circles represents the treatment line at hand. Numbers in parentheses indicate the number of replicate flies within a treatment. pD is a p-value for comparing differences in the distribution of within treatment phase values. pM is a p-value for comparing differences between average phase of treatments. M & O. phi represents average phase of a treatment. r represents average phase consistency of a treatment. p is a p-value resulting from the bivariate comparison of both groups taking phase and phase consistency into account.
Figure A. 26 | Analysis of PER-driven bioluminescence of a reporter with a 24693 male compared to a reporter co-housed with a wild-type conspecific using flies as replicates. All plotted measures have been defined and discussed in Section 4.3. The generation and analysis of all included measures have been performed as described in Section 4.4. The circular plots displayed in L to O have been performed according to (Levine et al., 2002b). Dif represents phase difference in hours. Asterisks represent controls whereas circles represent the treatment line at hand. Numbers in parentheses indicate the number of replicate flies within a treatment. pD is a p-value for comparing differences in the distribution of within treatment phase values. pM is a p-value for comparing differences between average phase of treatments. M & O, phi represents average phase of a treatment. r represents average phase consistency of a treatment. p is a p-value resulting from the bivariate comparison of both groups taking phase and phase consistency into account.
Figure A.27 | Analysis of PER-driven bioluminescence of a reporter with a 27790 male compared to a reporter co-housed with a wild-type conspecific using flies as replicates. All plotted measures have been defined and discussed in Section 4.3. The generation and analysis of all included measures have been performed as described in Section 4.4. The circular plots displayed in L to O have been performed according to (Levine et al., 2002b). L & N. Dif represents phase difference in hours. Asterisks represent controls whereas circles represents the treatment line at hand. Numbers in parentheses indicate the number of replicate flies within a treatment. pD is a p-value for comparing differences in the distribution of within treatment phase values. pM is a p-value for comparing differences between average phase of treatments. M & O, phi represents average phase of a treatment. r represents average phase consistency of a treatment. p is a p-value resulting from the bivariate comparison of both groups taking phase and phase consistency into account.
Figure A. 28 | Analysis of PER-driven bioluminescence of a reporter with a 34278 male compared to a reporter co-housed with a wild-type conspecific using flies as replicates. All plotted measures have been defined and discussed in Section 4.3. The generation and analysis of all included measures have been performed as described in Section 4.4. The circular plots displayed in L to O have been performed according to (Levine et al., 2002b). L & N. Dif represents phase difference in hours. Asterisks represent controls whereas circles represents the treatment line at hand. Numbers in parentheses indicate the number of replicate flies within a treatment. pD is a p-value for comparing differences in the distribution of within treatment phase values. pM is a p-value for comparing differences between average phase of treatments. M & O. phi represents average phase of a treatment. r represents average phase consistency of a treatment. p is a p-value resulting from the bivariate comparison of both groups taking phase and phase consistency into account.
Figure A. 29 | Analysis of PER-driven bioluminescence of a reporter with a 35938 male compared to a reporter co-housed with a wild-type conspecific using flies as replicates. All plotted measures have been defined and discussed in Section 4.3. The generation and analysis of all included measures have been performed as described in Section 4.4. The circular plots displayed in L to O have been performed according to (Levine et al., 2002b). L & N. Dif represents phase difference in hours. Asterisks represent controls whereas circles represents the treatment line at hand. Numbers in parentheses indicate the number of replicate flies within a treatment. pD is a p-value for comparing differences in the distribution of within treatment phase values. pM is a p-value for comparing differences between average phase of treatments. M & O. phi represents average phase of a treatment. r represents average phase consistency of a treatment. p is a p-value resulting from the bivariate comparison of both groups taking phase and phase consistency into account.
A.1.2.b OTHER CANDIDATES

Several other lines with mutations in candidate loci were screened. These included lines with mutations in Desaturase 1, Cyp6a20, Dysbindin ortholog (H. sapiens), Glial Lazarillo (Glaz), inactive, Neuroglian, OR83b, per, Pox neuro, Shaker, fruitless. Lines causing a significant change in the reporter’s PER expression compared to a reporter housed with a control, are shown below. One exception is per0 which does not show an effect but is included for discussion purposes.

Figure A. 30 | Analysis of PER-driven bioluminescence of a reporter with a Glaz mutant male compared to a reporter co-housed with a wild-type conspecific using flies as replicates. All plotted measures have been defined and discussed in Section 4.3. The generation and analysis of all included measures have been performed as described in Section 4.4. The circular plots displayed in L to O have been performed according to (Levine et al., 2002b). L & N. Dif represents phase difference in hours. Asterisks represent controls whereas circles represents the treatment line at hand. Numbers in parentheses indicate the number of replicate flies within a treatment. pD is a p-value for comparing differences in the distribution of within treatment phase values. pM is a p-value for comparing differences between average phase of treatments. M & O, phi represents average phase of a treatment. r represents average phase consistency of a treatment. p is a p-value resulting from the bivariate comparison of both groups taking phase and phase consistency into account.
Figure A. 31 | Analysis of PER-driven bioluminescence of a reporter with a Cyp620 mutant male compared to a reporter co-housed with a wild-type conspecific using flies as replicates. All plotted measures have been defined and discussed in Section 4.3. The generation and analysis of all included measures have been performed as described in Section 4.4. The circular plots displayed in L to O have been performed according to (Levine et al., 2002b). L & N. Dif represents phase difference in hours. Asterisks represent controls whereas circles represents the treatment line at hand. Numbers in parentheses indicate the number of replicate flies within a treatment. pD is a p-value for comparing differences in distribution of within treatment phase values. pM is a p-value for comparing differences between average phase of treatments. M & O. phi represents average phase of a treatment. r represents average phase consistency of a treatment. p is a p-value resulting from the bivariate comparison of both groups taking phase and phase consistency into account.
Figure A. 32 | Analysis of PER-driven bioluminescence of a reporter with a per\textsuperscript{D} male compared to a reporter co-housed with a wild-type conspecific using flies as replicates. All plotted measures have been defined and discussed in Section 4.3. The generation and analysis of all included measures have been performed as described in Section 4.4. The circular plots displayed in L to Q have been performed according to Levine et al., 2002b. L & N. Dif represents phase difference in hours. Asterisks represent controls whereas circles represent the treatment line at hand. Numbers in parentheses indicate the number of replicate flies within a treatment. pD is a p-value for comparing differences in the distribution of within treatment phase values. pM is a p-value for comparing differences between average phase of treatments. M & O. phi represents average phase of a treatment. r represents average phase consistency of a treatment. p is a p-value resulting from the bivariate comparison of both groups taking phase and phase consistency into account.
Appendix II  LOCOMOTOR ACTIVITY PHENOTYPES OF SELECT CANDIDATE LINES

Figure A. 33 | Analysis of locomotor activity of line 86-2nd compared to control in LD. All plotted measures have been defined, generated, and discussed in Section 5.3.a. The circular plots displayed in N & O have been performed according to (Levine et al., 2002b). N. Dif represents phase difference in hours. Asterisks represent controls whereas circles represents the treatment line at hand. Numbers in parentheses indicate the number of replicate flies within a treatment. p0 is a p-value for comparing differences in the distribution of within treatment phase values. pm is a p-value for comparing differences between average phase of treatments. phi represents average phase of a treatment. r represents average phase consistency of a treatment. p is a p-value resulting from the bivariate comparison of both groups taking phase and phase consistency into account.
Figure A. 34 | Analysis of locomotor activity of line 86-2nd compared to control in DD. All plotted measures have been defined, generated, and discussed in Section 5.3.a. The circular plots displayed in N & O have been performed according to (Levine et al., 2002b). N. Dif represents phase difference in hours. Asterisks represent controls whereas circles represents the treatment line at hand. Numbers in parentheses indicate the number of replicate flies within a treatment. pD is a p-value for comparing differences in the distribution of within treatment phase values. pM is a p-value for comparing differences between average phase of treatments. O. phi represents average phase of a treatment. r represents average phase consistency of a treatment. p is a p-value resulting from the bivariate comparison of both groups taking phase and phase consistency into account.
Figure A. 35 | Analysis of locomotor activity of line 298-2nd compared to control in LD. All plotted measures have been defined, generated, and discussed in Section 5.3.a. The circular plots displayed in N & O have been performed according to (Levine et al., 2002b). N. Dif represents phase difference in hours. Asterisks represent controls whereas circles represents the treatment line at hand. Numbers in parentheses indicate the number of replicate flies within a treatment. pD is a p-value for comparing differences in the distribution of within treatment phase values. pM is a p-value for comparing differences between average phase of treatments. O, phi represents average phase of a treatment. r represents average phase consistency of a treatment. p is a p-value resulting from the bivariate comparison of both groups taking phase and phase consistency into account.
Figure A.36 | Analysis of locomotor activity of line 298-2nd compared to control in DD. All plotted measures have been defined, generated, and discussed in Section 5.3.a. The circular plots displayed in N & O have been performed according to (Levine et al., 2002b). N, Dif represents phase difference in hours. Asterisks represent controls whereas circles represents the treatment line at hand. Numbers in parentheses indicate the number of replicate flies within a treatment. pD is a p-value for comparing differences in the distribution of within treatment phase values. pM is a p-value for comparing differences between average phase of treatments. O, phi represents average phase of a treatment. r represents average phase consistency of a treatment. p is a p-value resulting from the bivariate comparison of both groups taking phase and phase consistency into account.
Figure A. 37 | Analysis of locomotor activity of line 1036-X compared to control in LD. All plotted measures have been defined, generated, and discussed in Section 5.3.a. The circular plots displayed in N & O have been performed according to [Levine et al., 2002b]. N. Dif represents phase difference in hours. Asterisks represent controls whereas circles represents the treatment line at hand. Numbers in parentheses indicate the number of replicate flies within a treatment. pD is a p-value for comparing differences in the distribution of within treatment phase values. pM is a p-value for comparing differences between average phase of treatments. O. phi represents average phase of a treatment. r represents average phase consistency of a treatment. p is a p-value resulting from the bivariate comparison of both groups taking phase and phase consistency into account.
Figure A. 38 | Analysis of locomotor activity of line 1036-X compared to control in DD. All plotted measures have been defined, generated, and discussed in Section 5.3.a. The circular plots displayed in N & O have been performed according to (Levine et al., 2002b). N. Dif represents phase difference in hours. Asterisks represent controls whereas circles represent the treatment line at hand. Numbers in parentheses indicate the number of replicate flies within a treatment. pD is a p-value for comparing differences in the distribution of within treatment phase values. pM is a p-value for comparing differences between average phase of treatments. O, phi represents average phase of a treatment. r represents average phase consistency of a treatment. p is a p-value resulting from the bivariate comparison of both groups taking phase and phase consistency into account.
Appendix III  MATLAB CODES

MATLAB Code 1

```matlab
function [AllXps1MtrxNrm]=JA_nrmlz_each_run(CtrlLine,Line_W_All,Wells_All,varargin)
%This function will generate a 3D matrix with all experiments - experiment will be aligned later
%Each experiment gets internally normalized to its own controls by dividing all values by experiment-specific control mean.
%CtrlLine = name of control according to which normalization will be performed.
%Line_W_All = 2 column table for all lines and experiments that will be considered. Column 1 is Experiment name (e.g. Run046). Column 2 is Line name (e.g. Ctrl). There should be 1 row/line/exp %Wells ALL = table where each row has the list of wells of the line and exp corresponding to the same row in Line_W_All table.
%e.g. command: [AllXps1MtrxNrm]=nrmlz_each_Run('LFBG_F_CSisoX',Line_W_All,Wells_All,Run072TC2.f);
save('AllXps1MtrxNrm', 'AllXps1MtrxNrm');

%***order of entered files will match their order in the 3D file and should match the order in the Exps.mat file that will be used later.
exp_num=length(varargin); % get the # of experiments entered

% creating list of ctrls from all exps
Line_W_ctrls{:}={}, index_ctrl={}; Wells_ctrl={}; index_ctrl=find(strcmp(Line_W_All(:,2),CtrlLine)); % get the row index of all the ctrls
Wells_ctrl=Line_W_All(index_ctrl,:); % get names of all the ctrls (all the same but need to get them)

% get all the wells of the ctrls - 1 row of wells for each experiment - contains zeros because exp have unequal # of ctrl or mut wells

% combine all experiments into 1 3d matrix for easier future access - experiment times will be aligned elsewhere
AllXps1MtrxNrm{1,:}={};
AllXps1MtrxNrm(1:768,1:768,1:exp_num)=NaN; % in case 1st file is the shortest *** should be long enough to incorporate all experiments
for i=1:exp_num
    file{i}=varargin{i};
    AllXps1MtrxNrm{1:length(file{i}),1:length(file{i}),i}=file; % filling in each z layer with an experiment - unfilled cells will contain NaN to account for unequal exp lengths
end
for e=1:length(Line_W_ctrls{:}) % now go thru each exp
    ctrls={};
    ctrls=Wells_ctrl(:,find(Wells_ctrl(:,1)==e)); % consider ctrl well # and remove wells with 0 value (see above explanation about zeros)
    CPS_Ctrl_mean=nanmean(nanmean(AllXps1MtrxNrm{:,ctrs,e}));
    AllXps1MtrxNrm{:,ctrs,e}=AllXps1MtrxNrm{:,ctrs,e}/CPS_Ctrl_mean; % divide each value in the exp with exp ctrl mean
end
save('AllXps1MtrxNrm', 'AllXps1MtrxNrm');
```

MATLAB Code 2

```matlab
function [AllXps1MtrxNrm]=JAȂn_runs(ZT,AllXps1MtrxNrm,varargin)
%aligns all .times files to earliest file and places them in a new 3D matrix AllXps1MtrxNrm % also gives out table of aligned times for user to double check %AllXps1MtrxNrm=3D file generated by nrmlz_each_Run function %***order of entered .times files should match their order in the 3D file AllXps1MtrxNrm %e.g commands %[AllXps1MtrxNrm]=Align_Runs(ZT,AllXps1MtrxNrm,Run072TC2.time);
save('AllXps1MtrxNrm', 'AllXps1MtrxNrm');

%[AllXps1MtrxNrm]=3D file generated by nrmlz_each_Run function %***order of entered .times files should match their order in the 3D file AllXps1MtrxNrm %e.g commands %[AllXps1MtrxNrm]=Align_Runs(ZT,AllXps1MtrxNrm,Run053TC21600.time,Run055TC21600.time,Run058TC21600.time,Run059TC21600.time,Run069TC21600.time,Run070TC21600.time,Run071TC21600.time);
save('AllXps1MtrxNrm', 'AllXps1MtrxNrm');

cell{i}=[];
times(:,1:length(varargin))=0; % make a table of times for each exp aligned to visualize if alignment is working ** 500 should be enough for current length of exp - empties = zero
AllXps1MtrxNrm{1,1:768,1:768,1:length(varargin)}=i; % same for new 3D data file that will contain aligned data files
for i=1:length(varargin) % now go through each exp
    file{i}=varargin{i};
    cell{i}=file{i,1}; % grab 1st timepoint of each exp
```
MATLAB Code

function [rho2]=JA_bivariate_test_nrmlz(mean1,r1,mean2,r2,smmarkersize, ...
\lrgmarkersize,Color1,color2)
\% BIVARIATE Circular test for bivariate circular samples
\% [rho2]=bivariate(thet1,thol,th2a,th2b)
\% Hotelling's two sample test,
\% "tests whether the centers of the two samples
\% deviate significantly from each other"
\% Source: Batschelet 81, p. 150
\% \lrgmarkersize,Color1,color2

% Copyright (C) Jeffrey Hall Lab, Brandeis University. 
% Use and distribution of this software is free for academic 
% purposes only, provided this copyright notice is not removed. 
% Not for commercial use. 
% Unless by explicit permission from the copyright holder. 
% Mailing address: 
% Jeff Hall Lab, Kalman Bldg, Brandeis Univ, Waltham MA 02454 USA 
% Email: hall@brandeis.edu

end

m=find(cell1==min(cell1)); % find the file with the earliest 1st timepoint
file=mean1; file=varargin(m(1)); % consider the file with the earliest timepoint - m(1) incase more than 1
start at the same time
times(size(file,:),m(1))=file(:,i); % put the timepoints of that file in the times table in the
column that corresponds to the order the chosen exp has been entered in
b1[]=[]; b1=allxps1mtrxnm(); m>(); b1=bl=('is-nan(b1(:,i),):) % remove NaN from the file
Allxps1mtrxnmLNA(:,1:length(file(:,i),)=length(b1(:,i),))=m(); % put the cleaned file in

Allxps1mtrxnmLNA(:,1:length(file(:,i),)=length(b1(:,i),))=m(); % put the cleaned file in

Allxps1mtrxnmLNA(:,1:length(file(:,i),)=length(b1(:,i),))=m(); % put the cleaned file in

% ***** this should be better
end

end

% ***** this should be better - what if we have a reading at exactly midnight????!!!
times(all(times==0,)=[]); % remove rows in times where all values are 0
Allxps1mtrxnmLNA(:,1:length(times(:,i),)=length(allxps1mtrxnmLNA(:,i),));=[]; % empty those rows
from allxps1mtrxnmLNA as well
Allxps1mtrxnmLNA(:,1:length(times(:,i),)=length(allxps1mtrxnmLNA(:,i),));=[]; % empty those rows

% Make zeitgeber format
for i=length(times(:,i),)
time_ZT(r(i))=times(z,=isnan(times(z,)=0))); % get first non NaN value from each times row to
create one long time line that applies to all experiments
% time_ZT(r(i))=floor(time_ZT(r(i),=100)*rem(time_ZT(r(i),, 100)/60);
(time_ZT(r(i),,id)=min(Abs(time_ZT(r(i),,id)-time_ZT(r(i),,id)));
if time_ZT(r(i),=15
% time_ZT(r(i),=15;
else
\time_ZT(r,0)=time_ZT(r,)*2*idx,2)/2*idx,1);
end

save('Allxps1mtrxnmLNA', 'Allxps1mtrxnmLNA'); save('times', 'times'); save('time_ZT', 'time_ZT');

MATLAB Code 3
if
SSy1
SSx1
meany1
meanx1
y1
x1
n2
n1
%jade
end
end
if
analysis are affected by negative numbers
for
end
end
if
range from 0 to 12 and
means1_2
%jade
end
end
if
global
DAILY_HOURS
if
isempty(DAILY_HOURS)
DAILY_HOURS=24;
end
if
= (DAILY_HOURS==24)
DAILY_HOURS
end
global avghour_init
if
isempty(avaughour_init)
avaughour_init=[];
end
end
% converts a negative average to the format of 0 to 24
if
means1_2<0;
means1_2=means1_2+24; % converts negative #s to the format of 0 to 24 just in case
else
means1_2=means1_2-mean1_2;
end
end
% normalize by subtracting phase of ctrl
for
i=1:length(means1)
if
means1(i)-mean1_2<0; % normalize by subtracting phase of ctrl
means1(i)=means1(i)-mean1_2; % converts negative #s to the format of 0 to 24 just in case
else
means1(i)=means1(i)-mean1_2;
end
end
% This is avg of ctrl
meany1=mean(x1);
meanx1=mean(y1);
mean1=length(x1);
mean1=length(y1);
x1=x1.*cos(means1/DAILY_HOURS*pi);
y1=y1.*sin(means1/DAILY_HOURS*pi);
meanx2=mean(x2);
meany2=mean(y2);
mean2=length(x2);
mean2=length(y2);
x2=x2.*cos(means2/DAILY_HOURS*pi);
y2=y2.*sin(means2/DAILY_HOURS*pi);
meanx2=mean(x2);
meany2=mean(y2);
mean1=meanx1,meany1];
SSx1=sum((x1-meany1).^2); % This is the sum of squares of the deviations
SSy1=sum((y1-meanx1).^2); % This is the sum of squares of the deviations
C1 = mean((x1-meany1).*(y1-meanx1));
if
isempty(means2)
x2=x2.*cos(means2/DAILY_HOURS*pi);
y2=y2.*sin(means2/DAILY_HOURS*pi);
meanx2=mean(x2);
meany2=mean(y2);
mean2=length(x2);
mean2=length(y2);
x2=x2.*cos(means2/DAILY_HOURS*pi);
y2=y2.*sin(means2/DAILY_HOURS*pi);
meanx2=mean(x2);
meany2=mean(y2);
mean2=length(x2);
mean2=length(y2);
x2=x2.*cos(means2/DAILY_HOURS*pi);
y2=y2.*sin(means2/DAILY_HOURS*pi);
meanx2=mean(x2);
meany2=mean(y2);
mean2=length(x2);
mean2=length(y2);
x2=x2.*cos(means2/DAILY_HOURS*pi);
y2=y2.*sin(means2/DAILY_HOURS*pi);
meanx2=mean(x2);
meany2=mean(y2);
mean2=length(x2);
mean2=length(y2);
x2=x2.*cos(means2/DAILY_HOURS*pi);
y2=y2.*sin(means2/DAILY_HOURS*pi);
meanx2=mean(x2);
meany2=mean(y2);
mean2=length(x2);
mean2=length(y2);
x2=x2.*cos(means2/DAILY_HOURS*pi);
y2=y2.*sin(means2/DAILY_HOURS*pi);
meanx2=mean(x2);
meany2=mean(y2);
mean2=length(x2);
mean2=length(y2);
x2=x2.*cos(means2/DAILY_HOURS*pi);
y2=y2.*sin(means2/DAILY_HOURS*pi);
meanx2=mean(x2);
meany2=mean(y2);
mean2=length(x2);
mean2=length(y2);
x2=x2.*cos(means2/DAILY_HOURS*pi);
y2=y2.*sin(means2/DAILY_HOURS*pi);
meanx2=mean(x2);
meany2=mean(y2);
mean2=length(x2);
mean2=length(y2);
MATLAB Code

```matlab
confidence=0.01-fdist(Fvalue,Fn,Fm)*100;
end

cla
hold on
ul=plot(x1,y1,symbol1);
if ~isempty(means1)
ul=plot([x2,y2,symbol2];
{o=}plot([[ meanx1],[0 meany2],'-');
{o=}plot(meanx2,meany2,symbol2);
set(o{1},'LineWidth',lineWidth);
set(o{1},'LineWidth',lineWidth);
if ~isempty(color2)
set([u2,o{2}],o{2}','color',color2);
end
end
if nargin>4 & smmarkersize>0
set(u1,'MarkerSize',smmarkersize);
set(u2,'MarkerSize',smmarkersize);
end
if nargin>4 & lrgmarkersize>0
set(o{1},'MarkerSize',lrgmarkersize);
set(o{1},'MarkerSize',lrgmarkersize);
end
if ~isempty(means2)
[rh0,phi]=rect2pol(meanx1,meany1);
[rho2,phi2]=rect2pol(meanx2,meany2);
phi2=phi2/pi*DAILY_HOURS;
phi1=phi1/pi*DAILY_HOURS;
xlabel(sprintf('n o:r=%.1f  phi=%.1f
% n p=%.3f',
    rho1,phi1,rho2,phi2,(100-confidence)/100),'fontsize',6);
end
set(gca,'xlim',[-1.1 1.1], 'ylim',[-1.1 1.1]);
theth=1; %+1;
plot(cos(th),sin(th),'-', 'color', 'k');
axis square
set(gca,'xticklabel',[],'yticklabel',[]);
return
```

MATLAB Code 4

```matlab
function [h2]=MULTIPLE_TESTNSURL(s1,s2,fontsize,mk1,mk2,color1,color2)

MULTIPLE_TEST: HOURTEST combined with DISPERSION_TEST
m = multiple_test(s1,s2,[fontsize,mk1,mk2,color1,color2])

% Copyright (C) Jeffrey Hall Lab, Brandeis University.  
% Use and distribution of this software is free for academic  
% purposes only, provided this copyright notice is not removed.  
% Not for commercial use.  
% Unless by explicit permission from the copyright holder.  
% Mailing address:  
% Jeff Hall Lab, Kalman Bldg, Brandeis Univ, Waltham MA 02454 USA  
% Email: hall@brandeis.edu 

if nargin<3 | fontsize==0
    fontsize=[];
end
if nargin<5
    mk1=[];
end
if nargin<7
    mk2=[];
end
if nargin<9
    color1='b';
end
if nargin<11
    color2='b';
end
```
end
%jade % to normalize - where phase of ctrl is zero and everything is adjusted accordingly
s1 =1/2*avghour(s1); % This is avg of ctrl - Does avg of hour - where results range from 0 to 12 and -0.0000001 to -11.999999
if s1 <0;
s1 = s1+24; % converts a negative average to the format of 0 to 24
else
end
for i=1:length(s1)
if s1(i)<s1(i)-s1_2; % normalize by subtracting phase of ctrl
s1(i)=s1(i)-s1_2; % converts negative #s to the format of 0 to 24 just incase subsequent analysis
are affected by negative numbers
else s1(i)=s1(i)-s1_2;
end
end
for i=1:length(s2)
if s2(i)<s2(i)-s1_2; % normalize by subtracting phase of ctrl
s2(i)=s2(i)-s1_2; % converts negative #s to the format of 0 to 24 just incase subsequent analysis
are affected by negative numbers
else s2(i)=s2(i)-s1_2;
end
end
%Jade
cla
n1=length(s1);
n2=length(s2);
M=n1+n2;
v1=sum(cos(s1/24*pi));
v2=sum(cos(s2/24*pi));
w1=sum(sin(s1/24*pi));
w2=sum(sin(s2/24*pi));
V=v1+v2;\nW=w1+w2;\nR1=sqrt(v1^2+w1^2);\nR2=sqrt(v2^2+w2^2);\nM=sqrt(V^2+4*W^2);\ns12e=(n1*R1)/n1;\ns22e=(n2*R2)/n2;
% hourtest\nF=(N-1)*((R1+R2)-R)/(N-R1-R2);\nx=(R1*R2)/(n1*n2);\n% F=(n2-1)*(n1-1)/(n2-2)*(n1-2);\nu1=m1;\nu2=m2;\nif (F<1)\nF=F/1;\ntmp=m2;\nu1=m1;\nu1=tmp;
end
\fprintf('F=%f is distributed as F(%d,%d)\n',F,u1-1,u2-1);
\plot(cos(s1/24*pi)),sin(s1/24*pi),',b.';
\hold on\n\plot(cos(s2/24*pi)),sin(s2/24*pi),',r.';
\plot(v1,w1,+',');\n\plot(v2,w2,','o');\n\plot(V,W,','k');
figure\nglobal avghour init
old=avghour init;
avhour init=[];

hpolar(0,2.0,'w.',0,1,fontsize);
\set(hpolar,'color','white');\nhold on\n\text{th}=0:pi/40:pi;\nx=cos(th);\ny=sin(th);\nplot(x,y,':k','LineWidth',1.5);\nplot(0.5*x, 0.5*y, ':k');
\plot([-1 1],[0 0],'k-');\n\plot([0 0],[-1 1],'k-');\nsym1="'";\nsym2="o";
h1=avghour(s1,sym1,1.35,fontsize,mk1,mk2,color1);
MATLAB Code 5

function [GroupP2, dataP2, var_col_num_Labels] = JA_cclct_all_vrs(CtrlLine, TrtLine, dummy, p, Line_W_All, Wells_All, Exps, AllXpsMttrNmrAlnd, times)
%Calculates many variables from luc-analyze and peakcirclip from normalized data. Output values from
%peakcirclip are also normalized - see thesis
%CtrlLine=Name of line considered control
%TrtLine=Name of line
%dummy=user provided file - ask Jade
%Line_W_All = 2 column table for all lines and experiments that will be considered. Column 1 is Experiment
%name (e.g. Run046). Column 2 is Line name (e.g. Ctrl). There should be 1 row/line/exp
%Wells_ALL = table where each row has the list of wells of the line and exp corresponding to the same row in
%Line_W_All table.
%Exps = Table with 2 columns - 2nd is name of each experiment and 1st is experiment number (i.e. 1 for first
%experiment)
%AllXpsMttrNmrAlnd = File generated by Aln_Runs function
%times = File generated by Aln_Runs function

var_col_num_Labels = {'Exps#'; 'Well#'; 'Normalized Mean Counts/Hr'; 'SEM Counts/Sec' 'Mean Normalized Counts/Hr';
'SEM Normalized Counts/Hr'; 'Period (Autocorrelation)' 'Rhythmicity Index' 'Rhythm Strength' 'Period(Mesa)';
'Phase' 'Phase Consistency'; 
'Normalized Mean Counts/Hr'; 'Mean Normalized Counts/Hr' 'SEM Counts/Sec' 'SEM Normalized Counts/Hr'; 'Period (Autocorrelation)' 'Rhythmicity Index' 'Rhythm Strength' 'Period(Mesa)';
var_col_num_Labels = 

Lines = []; 
if strcmp(CtrlLine, {'All'})
Lines = [TrtLine; Lines];
else
Lines = [Lines, CtrlLine];
end

for i=1:length(Lines)
Lines(i); L
% creating list of mutant wells to be analyzed
Line_W_mut{i}; index_mut{i}; Wells_mut{i};
index_mut{i}=find(strcmp(Line_W_All(:,2),Lines(i)));
Line_W_mut{index_mut{i}}=Line_W_All(index_mut{i},:);
Wells_mut{index_mut{i}}=Wells_All(index_mut{i},:);

% creating list of ctrl wells to be analyzed
Line_W_ctrl{i}; index_ctrl{i}; Wells_ctrl{i};
index_ctrl{i}=find(strcmp(Line_W_All(:,2),CtrlLine));
Line_W_ctrl{index_ctrl{i}}=Line_W_All(index_ctrl{i},:);
Wells_ctrl{index_ctrl{i}}=Wells_All(index_ctrl{i},:);

% considering only ctrls from experiments where mutant in question is studied
index_ctrl{i};
for i=1:length(Line_W_ctrl{i})
if sum(strcmp(Line_W_mut{i},Line_W_ctrl{i}))==0
else index_ctrl{i}={}; c=c+1;
end
end
Line_W_ctrl{index_ctrl{i}};
Wells_ctrl{index_ctrl{i}};

for e=1:length(Line_W_ctrl{i}) %now go thru each mut exp
ctrls{e}; muts{e};

ctrls{e}=Wells_ctrl{e}, find(Wells_ctrl{e}==1);
mutss{e}=Wells_mut{e}, find(Wells_mut{e}==1);
end

for e=1:length(mutss{e}) %figure which dimension the appropriate exp data
end
MATLAB Code 6

```matlab
function [TableFliesCPS, TableLabelsFliesCPS] = JA_cps_cmn_flis(CtrlLine, TrtLine, nrand, Line_W_All, Wells_All, Exps, AllXps1MtrxNrmAlnd)
% creates means for CPS time series using flies as replicates but uses re-sampling method – see thesis
% CtrlLine = name of the control
% TrtLine = Name of line
% Line_W_All = 2 column table for all lines and experiments that will be considered. Column 1 is Experiment name (e.g. Run046). Column 2 is Line name (e.g. Ctrl). There should be 1 row/line/exp
% Wells_ALL = table where each row has the list of wells of the line and exp corresponding to the same row in Line_W_All table
% Exps = Table with 2 columns – 2nd is name of each experiment and 1st is experiment number (i.e. 1 for first experiment)
% nrand = # of randomization – apparently 10000 is acceptable
% AllXps1MtrxNrmAlnd = is the 3D Matrix of all the experiments at hand normalized and aligned by previous functions

exp_num = length(AllXps1MtrxNrmAlnd(:, 1, :)); % get # of experiments inputted

Lines = ();
if strcmp(TrtLine, 'All')
    Lines(:, 1) = TrtLine;
    Lines(:, 2) = CtrlLine;
else
    Lines = unique(Line_W_All(:, 2));
end
```
% make a large table. 8 columns/line. Lines back to back horizontally / each 8 contain Avg, SEM, n of flies, & n of experiments for ctrl & line
% note that the control values for each line will not be identical because a subset with eual number of flies is chosen for each line and this will be explained more later % 2 tables - 1 is for the labels and the other for values TableLabelsFliesCPS=[]; TableCPSFlies=[];
TableLabelsFliesCPS(:,1:(length(Lines)*1))='x'; % Table of Labels TableCPSFlies(:,:,1:(length(Lines)*1))='x'; % Table of Labels TableCPSFliesCPS=length(Allxps1MtrxNrmAlnd(:,1,:)),1:(length(Lines)*1))=NaN; % Table of Values #rows = # of available time points

for L=1:length(Lines) % go thru each line Line=Lines(L);

% creating list of mutant wells to be analyzed
Line_W_mut=[]; indx_mut=[]; Wells_mut=[];
Line_W_mut=Line_W_All(find(Line_W_All(:,1),Line));
Wells_mut=Wells_All(find(Line_W_All(:,1),:));
% creating list of ctrl wells to be analyzed
Line_W_ctrl=[]; indx_ctrl=[]; Wells_ctrl=[];
indx_ctrl=find(Line_W_ctrl(:,1),Line_W_All);
Line_W_ctrl=Line_W_All(find(Line_W_ctrl(:,1),:));
Wells_ctrl=Wells_All(find(Line_W_ctrl(:,1),:));
% considering only ctrls from experiments where mutant i
% make a large table. 8 columns/line. Lines back to back horizontally / each 8 contain Avg, SEM, n of flies, & n of experiments for ctrl & line
% note that the control values for each line will not be identical because a subset with eual number of flies is chosen for each line and this will be explained more later % 2 tables - 1 is for the labels and the other for values TableLabelsFliesCPS=[]; TableCPSFlies=[];
TableLabelsFliesCPS(:,1:(length(Lines)*1))='x'; % Table of Labels TableCPSFlies(:,:,1:(length(Lines)*1))='x'; % Table of Labels TableCPSFliesCPS=length(Allxps1MtrxNrmAlnd(:,1,:)),1:(length(Lines)*1))=NaN; % Table of Values #rows = # of available time points

for L=1:length(Lines) % go thru each line Line=Lines(L);

% creating list of mutant wells to be analyzed
Line_W_mut=[]; indx_mut=[]; Wells_mut=[];
Line_W_mut=Line_W_All(find(Line_W_All(:,1),Line));
Wells_mut=Wells_All(find(Line_W_All(:,1),:));
% creating list of ctrl wells to be analyzed
Line_W_ctrl=[]; indx_ctrl=[]; Wells_ctrl=[];
indx_ctrl=find(Line_W_ctrl(:,1),Line_W_All);
Line_W_ctrl=Line_W_All(find(Line_W_ctrl(:,1),:));
Wells_ctrl=Wells_All(find(Line_W_ctrl(:,1),:));

% considering only ctrls from experiments where mutant in question is studied
indx_ctrl=[]; c=1;
for i=1:length(Line_W_ctrl(:,1))
    if sum(strcmp(Line_W_mut(:,1),Line_W_ctrl(:,1)))==0;
        indx_ctrl(i)=c;
        c=c+1;
    end
e
Line_W_ctrl=Line_W_ctrl(indx_ctrl,:);
Wells_ctrl=Wells_ctrl(indx_ctrl,:);
end Line_W_ctrl=Line_W_ctrl(indx_ctrl,:);
Wells_ctrl=Wells_ctrl(indx_ctrl,:);

% the following process will be carried out an 'nrand' # of times
fctrlFliesAvg=[]; fmutFliesAvg=[]; fctrlFliesSEM=[]; fmutFliesSEM=[];
for n=1:nrand
    fctrl=Allxps1MtrxNrmAlnd(:,randsample(ctrls,length(muts)),x); % pick random subset ctrl for this exp, and get their hrly data. m = # of mutants for this exp *** without replacement
    fmut=Allxps1MtrxNrmAlnd(:,randsample(muts,length(muts)),x); % take all mutants
    end
    if we have more than one experiment for this line, fctrl=fctrln(:,fctrl,AllxpsMtxrNrmAlnd(:,randsample(ctrls,length(muts)),x))
        % make a table of all ctrl flies data
        fmut=cat(2,fmut,AllxpsMtxrNrmAlnd(:,muts,x)); % & for mutants
        end
    else % if we have more mutants than controls - rarely
    if n==1 % if we have only one experiment for this line
        fctrl=AllxpsMtxrNrmAlnd(:,randsample(ctrls,length(muts)),x); % pick random subset ctrl for this exp, and get their hrly data. m = # of mutants for this exp *** without replacement
        fmut=AllxpsMtxrNrmAlnd(:,randsample(muts,length(muts)),x); % take all mutants
    end
        % make a table of all ctrl flies data
        fctrl=fctrln(:,fctrl,AllxpsMtxrNrmAlnd(:,ctrls,x)); % & for controls
        end

    else % if we have more than one experiment for this line, fctrl=fctrln(:,fctrl,AllxpsMtxrNrmAlnd(:,randsample(ctrls,length(muts)),x))
        % make a table of all ctrl flies data
        fctrl=fctrln(:,fctrl,AllxpsMtxrNrmAlnd(:,ctrls,x)); % & for controls
        end
    end % by the end, only one of the above scenarios would have applied and we end up with 2 data tables, one for each ctrl and one for each mut fly both tables have an equal number of flies
end fctrlAvg=[]; fmutAvg=[]; fctrlSEM=[]; fmutSEM=[]; fctrln=[]; fmutn=[];
% create 1 column containing avg (for each hr) & one for SEM & one for n for all flies from all exp (for ctrl)
fctrlAvg=nanmean(fctrl,2); fctrln=nanstd(fctrl,0,2)./sqrt(fctrln);
% & for mutants fmutAvg=nanmean(fmut,2); fmutn=nanstd(fmut,0,2)./sqrt(fmutn);
% now this mean of all flies is based on 1 round of random sampling from ctrl from each exp.
% So we will re-create this many times based on re-sampling (better representation)
if n==1; % store the data from the 1st round of sampling in 1st column of tables below
function [TableFliesCPS,TableLabelsFliesCPS,avg_row_Labels,TableLabelsFliesVars,avg_row_Labels]=JA_vrs cmbn_fls(TrtLine,TrtLine,nrand,GroupF2,dataF2)
% creates means and p-values for all variables using flies as replicates but uses re-sampling method - see thesis
% TrtLine = the name of the control
% TrtLine = the name of the control
% GroupF2 = 2 column table for all lines and experiments that will be considered. Column 1 is Experiment name (e.g. Run046). Column 2 is Line name (e.g. Ctrl). There should be 1 row/fly/line/exp
% nrand = #of randomization - apparently 10000 is acceptable

Lines={};
if strcmp(TrtLine,'All')
    Lines(1)=TrtLine;
    Lines(2)=TrtLine;
else
    Lines=unique(GroupF2(:,2));
end

TableFliesVars{}; TableLabelsFliesVars{};
TableFliesVars({;,(Length(Lines)*2)})=('x');
TableFliesVars({;,(Length(Lines)*2)})=('x');
TableFliesVars({;,(Length(Lines)*4)})=-8000;

var_row_Labels({;,:})=['Normalized Mean Counts/Hr' 'SEM Counts/Sec' 'Mean Normalized Counts/Hr' 'SEM
Normalized Counts/Hr' 'Period (Autocorrelation)' 'Rhythmicity Index' 'Rhythm Strength' 'Period(Mean)'
'Phase' 'Phase Consistency' '{(Last 3 Days)'} '{(Last 2 Days)'} '{(Last Day)'}
for var_col_num=1:15
    var_col_num
dataF2{2}=GroupF2{}
dataF2=dataF2(find(dataF2(:,var_col_num)>-1000),:); % eliminate individuals who matlab failed to calculate all variables for
GroupF2=GroupF2(find(dataF2(:,var_col_num)>-1000),:);
for i=1:Length(Lines)
    GroupF_mut{i}=[]; dataF_mut=i; index_mut=i;
    index_mut=find(strcmp(GroupF2(:,2),Line{i}));
    GroupF_mut{GroupF2(index_mut,:)};% got all mutant flies from all exp
    dataF_mut=dataF2{index_mut,:};
    GroupF_ctrl{i}=[]; dataF_ctrl{i}=index_ctrl=i;
    index_ctrl=find(strcmp(GroupF2(:,2),Line{i}));
    GroupF_ctrl=GroupF2(index_ctrl,:);% got all ctrls flies from all exp
    dataF_ctrl=dataF2{index_ctrl,:};
end
end

save('TableFliesCPS','TableLabelsFliesCPS');save('TableLabelsFliesCPS','TableLabelsFliesCPS');
% CtrlLine = the name of the control
% creates means for CPS time series using experiments as replicates

CtrlLine = 1
for r = 1:nrand
    GroupF_ctrl_flies = GroupF_mut_flies = dataF_ctrl_flies = dataF_mut_flies = [];
    for i = 1:length(ExpList)
        idx_ctrl_exp = find(strcmp(GroupF_ctrl(:,1), ExpList(i))); % index of controls in that exp
        idx_mut_exp = find(strcmp(GroupF_mut(:,1), ExpList(i))); % index of mutants in that exp
        if length(idx_ctrl_exp) > length(idx_mut_exp) % if we have more ctrls than experimentals - almost always is the case
            idx_ctrl_exp = randsample(idx_ctrl_exp, length(idx_mut_exp)); % get random control sample
            idx_ctrl_exp = idx_ctrl_exp(idx_ctrl_exp); % indx of mutants in that exp
        else % otherwise (more experimentals) get random sample from them
            idx_mut_exp = randsample(idx_mut_exp, length(idx_ctrl_exp));
            idx_ctrl_exp = idx_ctrl_exp(idx_ctrl_exp);
        end
        if i == 1 % if this is the first experiment, document it
            GroupF_ctrl_flies = GroupF_ctrl(idx_ctrl_exp, :); % ctrl flies
            dataF_ctrl_files = dataF_ctrl(idx_ctrl_exp, :);
            dataF_ctrl_files = dataF_ctrl(idx_ctrl_exp, :);
            GroupF_mut_flies = GroupF_mut(idx_mut_exp, :); % mut flies
            dataF_mut_files = dataF_mut(idx_mut_exp, :);
        else % if these are following experiments, concatenate them with the 1st
            GroupF_ctrl_flies = [GroupF_ctrl_flies, GroupF_ctrl(idx_ctrl_exp, :)];
            dataF_ctrl_files = [dataF_ctrl_files, dataF_ctrl(idx_ctrl_exp, :)];
            dataF_ctrl_files = [dataF_ctrl_files, dataF_ctrl(idx_ctrl_exp, :)];
        end
        Avg_var_ctrl_rnds(r) = mean(dataF_ctrl_flies(:, var_col_num)); % made averages of all collected ctrl flies
        n_var_ctrl_rnds = length(dataF_ctrl_flies(:, var_col_num)); % freq of columns in all dataF
        Avg_var_ctrl_rnds(r) = std(dataF_ctrl_flies(:, var_col_num)) / sqrt(length(dataF_ctrl_flies(:, var_col_num))); % avg of all randomizations
        Avg_var_ctrl_rnds(r) = mean(dataF_ctrl_files(:, var_col_num)); % mean of all mutants flies
        n_var_ctrl_rnds = length(dataF_ctrl_files(:, var_col_num)); % freq of columns in all dataF
        Avg_var_ctrl_rnds(r) = std(dataF_ctrl_files(:, var_col_num)) / sqrt(length(dataF_ctrl_files(:, var_col_num))); % made averages of all collected ctrl flies
        n_var_ctrl_rnds = length(dataF_ctrl_files(:, var_col_num)); % freq of columns in all dataF
        % once done, average randomizations
        Avg_var_ctrl = mean(Avg_var_ctrl_rnds); % avg of all randomizations
        SEM_var_ctrl = mean(SEM_var_ctrl_rnds);
        Avg_var_mut = mean(Avg_var_mut_rnds); % avg of all randomizations
        SEM_var_mut = mean(SEM_var_mut_rnds);
        n_var_mut = n_var_mut_rnds(1);
        % Calculate p-values on final means
        std_mut = SEM_var_ctrl * sqrt(n_var_ctrl);
        std_ctrl = SEM_var_mut * sqrt(n_var_mut);
        t = (Avg_var_ctrl - Avg_var_mut) / sqrt(((n_var_ctrl-1) + (n_varMut-1)) * std_ctrl^2 / n_var_ctrl + std_mut^2 / n_var_mut);
        p_Value = 2 * tcdf(abs(t), n_var_ctrl + n_var_mut - 2);
    end
end
% create a table with all necessary values
TableLabelsFliesVars{:,(1:*)} = 'Exp';
TableLabelsFliesVars{:,(2:*)} = 'p-Value';
TableLabelsFliesVars{:,(3:*)} = 'Avg_var_ctrl';
TableLabelsFliesVars{:,(4:*)} = 'SEM_var_ctrl';
TableLabelsFliesVars{:,(5:*)} = 'Avg_var_mut';
TableLabelsFliesVars{:,(6:*)} = 'SEM_var_mut';
TableLabelsFliesVars{:,(7:*)} = 'avg var mut';
TableLabelsFliesVars{:,(8:*)} = 'n_var_mut';
TableLabelsFliesVars{:,(9:*)} = 'length(ExpList)';
end
save('TableFliesVars','TableFliesVars');save('TableLabelsFliesVars','TableLabelsFliesVars');
%TrtLine=Name of line
%Line_W_All = 2 column table for all lines and experiments that will be considered. Column 1 is Experiment name (e.g. Run046). Column 2 is Line name (e.g. Ctrl). There should be 1 row/line/exp
%Wells_ALL = table where each row has the list of wells of the line and exp corresponding to the same row in %Line_W_All table.
%Exps = Table with 2 columns – 1st is name of each experiment and 1st is experiment number (i.e. 1 for first experiment)
%AllXps1MtrxNrmAlnd = is the 3D Matrix of all the experiments at hand normalized and aligned by previous functions

exp_num=length(AllXps1MtrxNrmAlnd(:,1,:)); % get # of experiments inputted

Lines=[];
if strcmp(TrtLine,'All')==1
   Lines=TrtLine;
else
   Lines=unique(Line_W_All(:,2));
end

TableLabelsExpCPS=[]; TableExpCPS=[];
TableLabelsExpCPS(:,1:(length(Lines)*1))='x'; % Avg SEM & n for ctrl & line for each line
TableLabelsExpCPS(:,1:(length(Lines)*1))='x';
TableExpCPS=length(AllXps1MtrxNrmAlnd(:,1,:)),1:(length(Lines)*1))=NaN; % # rows = # of available time points

for L=1:length(Lines) % go thru each line
   Line=Lines(L);
   
   % creating list of mutant wells to be analyzed
   Line_W_mut=[]; ind_mut=[]; Wells_mut=[];
   ind_mut=find(strcmp(Line_W_All(:,2),Line));
   Line_W_mut=Line_W_All(ind_mut,:);
   Wells_mut=Wells_All(ind_mut,:);
   
   % creating list of ctrl wells to be analyzed
   Line_W_ctrl=[]; ind_ctrl=[]; Wells_ctrl=[];
   ind_ctrl=find(strcmp(Line_W_All(:,2),CtrlLine));
   Line_W_ctrl=Line_W_All(ind_ctrl,:);
   Wells_ctrl=Wells_All(ind_ctrl,:);
   
   % considering only ctrl s from experiments where mutant in question is studied
   ind_ctrl=[};
   for i=1:length(Line_W_ctrl(:,1))
      if sum(strcmp(Line_W_ctrl(:,1),Line_W_ctrl(:,1)))==0;
         else ind_ctrl(i)=i; cc=cc+1;
      end
   end

   Line_W_ctrl=Line_W_ctrl(ind_ctrl,:);
   Wells_ctrl=Wells_ctrl(ind_ctrl,:);

   f_ctrl_Avg=[]; f_mut_Avg=[];
   for e=1:length(Line_W_ctrl(:,1)) %now go thru each mut exp
      f_ctrl=[]; f_mut=[];
      ctrl=[]; mut=[];
      ctrl=Wells_ctrl(e,find(Wells_ctrl(e,:)==0)); % remove (previously removed wells that were assigned as zero) to make list of ctrl and mut wells for this experiment
      mut=Wells_mut(e,find(Wells_mut(e,:)==0));
      indx_ctrl=find(strcmp(Exps(:,1),Line_W_ctrl(e,:))); % figure which dimension the appropriate exp data file is sitting in
      f_mut=AllXps1MtrxNrmAlnd(:,mut,x); % take all mutants (mutans always less than controls)
      f_ctrl=AllXps1MtrxNrmAlnd(:,ctrls,x);
      if ~isempty(f_ctrl_Avg)
         f_ctrl_Avg=nanmean(f_ctrl);
         f_mut_Avg=nanmean(f_mut);
      else
         f_ctrl_Avg=cat(2,f_ctrl_Avg,mean(f_ctrl,2)); % table - each column has hrly avgs from each exp for controls
         f_mut_Avg=cat(2,f_mut_Avg,mean(f_mut,2)); % table for mutants
      end
   end
   if ~isempty(f_ctrl_Avg)
      f_ctrl_AvgAvg=f_ctrl_Avg;
      f_ctrl_AvgSEM=f_ctrl_Avg;
      f_ctrl_AvgSEM=f_ctrl_Avg;
      f_ctrl_AvgSEM=f_ctrl_Avg;
      f_ctrl_AvgSEM=f_ctrl_Avg;
   end
   
   if ~isempty(f_mut_Avg)
      f_mut_Avg=min(f_mut_Avg);
      f_mut_Avg=max(f_mut_Avg);
MATLAB Code 9

```matlab
function [TableExpVars,TableLabelsExpVars] = JA_vrs_cmbn_xps(CtrlLine,TrtLine,GroupF2,dataF2)
% creates means and p-values for all variables using experiments as replicates - see thesis
% CtrlLine = name of the control
% TrtLine = Name of line
% GroupF2 = 2 column table for all lines and experiments that will be considered. Column 1 is Experiment name (e.g. Run046). Column 2 is Line name (e.g. Ctrl). There should be 1 row/fly/line/exp
% dataF2 = table where each row has the list of variables calculated from each fly (variables are shown in var_col_num_Labels file)

Lines{};
if strcmp(TrtLine,'All')
    Lines{}=TrtLine;
else
    Lines=unique(GroupF2(:,2));
end
TableLabelsExpVars{};
TableExpVars{}(:,length(Lines){})={'x'};
TableExpVars{}(:,length(Lines){})=0000;
for var_col_num=3:15
    var_col_num
    dataF2{}; GroupF2{};
dataF2{}=find(find(dataF{}(:,var_col_num)>1000),);
    GroupF2{}=find(find(dataF{}(:,var_col_num)>-1000),);
    for i=1:length(Lines)
        Lines{i}=
        GroupF{};
        idx_mut{};
        index_mut=find(find(GroupF{}(:,1),Lines{i}));
        GroupF{}=GroupF{}(index_mut,:);
        GroupF{};
        index_ctrl=find(find(GroupF{}(:,1),CtrlLine));
        GroupF{}=GroupF{}(index_ctrl,:);
        ExpList{};
        ExpList=unique(GroupF{});
        Avg_mut_Exps{};
        Avg_ctrl_Exps{};
        for i=1:length(ExpList)
            idx_ctrl_exp{}; idx_mut_exp{};
            idx_mut_exp = find(find(GroupF{}(:,1),ExpList{i}));
            idx_ctrl_exp = find(find(GroupF{}(:,1),ExpList{i}));
            idx_mut{};
            index_ctrl=unique(idx_ctrl_exp);
            index_mut=find(idx_mut_exp);
            index_mut=idx_mut_exp;
            else
                index_mut=idx_mut_exp;
                index_ctrl=idx_ctrl_exp;
            end
            GroupF{};
            dataF{};
            dataF{};
            dataF{};
            dataF{};
            Avg_mut_Exps{};
            Avg_ctrl_Exps{};
    end
    Avg_var_ctrl=mean(Avg_ctrl_Exps);
    SEM_var_ctrl=std(Avg_ctrl_Exps)/sqrt(length(Avg_ctrl_Exps));
    n_var_ctrl=length(Avg_ctrl_Exps);
    Avg_var_mut=mean(Avg_mut_Exps);
    SEM_var_mut=std(Avg_mut_Exps)/sqrt(length(Avg_mut_Exps));
    n_var_mut=length(Avg_mut_Exps);
end
```

Note: The MATLAB code provided is a function that creates mean and p-values for all variables using experiments as replicates. It takes 4 inputs: CtrlLine, TrtLine, GroupF2, and dataF2. It outputs 2 tables: TableExpVars and TableLabelsExpVars.
% Calculate p-values on final means
std_ctrlSEM_var_ctrl = sqrt(n_var_ctrl);
std_mut = SEM_var_mut * sqrt(n_var_mut);
t = (AVG_var_ctrl - AVG_var_mut) / (sqrt((n_var_ctrl - 1) * std_ctrl^2 + (n_var_mut - 1) * std_mut^2))^\(1/2\);
p_value = tcdf(abs(t), (n_var_ctrl + n_var_mut - 2));

% create a table with all necessary values
TableExpVars = {'TableExpVars'};
TableLabelsExpVars = {'TableLabelsExpVars'};

% create artificial damn load file for each line
for Lines
end

% create table with updated lines, monitors, & channels & save them
M15LD, M16LD, M17LD = JA_loc_clclt_all_vrs('LCSisoX', ZT, mon_names_DD, Fly_Lns, Fly_Mntrs, Fly_Chns, LD, varargin)

% create artificial damn load file for each line
file = varargin{1};
Mdummy = file;
c =;
while c < length(lins_fly_mrtr_chnls(:,1)) % go accross 1st row of table line by line (2 columns/line)
    Mdummy.data{1}{1}{c} = cell2mat(lins_fly_mrtr_chnls{1}{c})
    for i = 1:length(Mdummy.names{1}{c})
        Mdummy.names{1}{c}{i} = strcat( num2str{i}, cellstr(lins_fly_mrtr_chnls{1}{c}))
    end
    for r = 2:length(lins_fly_mrtr_chnls{1}{c})
        if cell2mat(lins_fly_mrtr_chnls{r}{c})
            file =;
            mon_num = lins_fly_mrtr_chnls{r}{c};
            mon_num = cell2mat(mon_num);
            file_name = strcat('M', num2str{mon_num}{char(1)});
            index_file = find(strcmp(mon_name, file_name));
            file varargin{index_file} = Mdummy.data{1}{r}{c} = file.data{1}{r}{c} = cell2mat(lins_fly_mrtr_chnls{r}{c});
    end
end
Mdummy = Mdummy.data;
eval(\{'lins_fly_mrtr_chnls\{1}{c} \_char(1) ',\'} = Mdummy;');
% Normalize
 eval(['LCSOR=mean(mean(' CtrlLine '_' char(LD) '.data(:,1:6)))']);
 eval(['SCSOR=mean(mean(' CtrlLine '.data(:,7:10)))']);
 eval(['CSOR=mean(mean(' CtrlLine '.data))']);

% table of zeitgeber times
 eval(['Mdummy=' lns_fly_mntr_chnls{1,1} '_' char(LD) '']);
time_ZT=[];
time_ZT=Mdummy';
for r=1:length(time_ZT)
 t = time_ZT(r);
 if t > 23.999999
  time_ZT(r)=t;
 end
 time_ZT(r)=time_ZT(r-1)+t(1:length(t)/60);
end
 eval(['time_ZT' '_' char(LD) '']);

% to run damn panels for each line and get data to plot time series
c=m;
 lns_cnt_Avg_SEM_lbls={}; lns_cnt_Avg_SEM={};
 while c<length(lns_fly_mntr_chnls{1,1})
  if c<=33*2 | ( c>48*2 & c<=53*2)
    eval(['save(''' lns_fly_mntr_chnls{1,c} '_' char(LD) '.data(:,1:5)/LCSOR*CSOR']);
    eval(['save(''' lns_fly_mntr_chnls{1,c} '_' char(LD) '.data(:,6:15)/SCSOR*CSOR']);
    eval(['save(''' lns_fly_mntr_chnls{1,c} '_' char(LD) '.data=' lns_fly_mntr_chnls{1,c} '_char(LD) '.data/SCSOR*CSOR']);
  else
    if c==48*2
      eval(['save(''' lns_fly_mntr_chnls{1,c} '_' char(LD) '.data=' lns_fly_mntr_chnls{1,c} '_char(LD) '.data(:,7:10)/SCSOR*CSOR']);
    else
      if c==54*2
        eval(['save(''' lns_fly_mntr_chnls{1,c} '_' char(LD) '.data=' lns_fly_mntr_chnls{1,c} '_char(LD) '.data(:,7:10)/SCSOR*CSOR']);
      else
        if c==55*2
          eval(['save(''' lns_fly_mntr_chnls{1,c} '_' char(LD) '.data=' lns_fly_mntr_chnls{1,c} '_char(LD) '.data(:,1:6)/LCSOR*CSOR');
        else
          if c==48*2
            eval(['save(''' lns_fly_mntr_chnls{1,c} '_' char(LD) '.data=' lns_fly_mntr_chnls{1,c} '_char(LD) '.data/SCSOR*CSOR');
          else
            eval(['save(''' lns_fly_mntr_chnls{1,c} '_' char(LD) '.data=' lns_fly_mntr_chnls{1,c} '_char(LD) '.data(:,1:5)/LCSOR*CSOR');
          end
        end
      end
    end
  end
end

while c<length(lns_fly_mntr_chnls{1,:}) % go accross 1st row of table line by line (2 columns/line)
 c=c+2;
end

% end
eval([ 'Mdummy'= lns_fly_mntr_chnls(:,c) ' ' char(LD) ' ']);
%damalyze_par = p.plotcol=[‘acto’ ‘flyf’ ‘auto’ ];
figure;
{filterf,SEM,meanff,px_v,py,ridex}=JA_loc_dam_panels(Mdummy,:,:length(Mdummy.data(:,::)),1,1,p);
saveas(gcf, ['dam Panels ' lns_fly_mntr_chnls(:,c) ' ' char(LD) ' '1] , ‘fig’); saveas(gcf, ['dam Panels ' lns_fly_mntr_chnls(:,c) ' ' char(LD) ' '1] , ‘jpg’);
close all;
%damalyze_par = p.plotcol=[‘acto’ ‘flyf’ ‘auto’ ‘pgram’ ];
figure;
{filterf,SEM,meanff,px_v,py,ridex}=JA_loc_dam_panels(Mdummy,:,:length(Mdummy.f(:,::)),1,1,p);
saveas(gcf, ['dam Panels ' lns_fly_mntr_chnls(:,c) ' ' char(LD) ' '2] , ‘fig’); saveas(gcf, ['dam Panels ' lns_fly_mntr_chnls(:,c) ' ' char(LD) ' '2] , ‘jpg’);
close all;
lns_cnt_avg_SEM_lbls(:,cc+:)=lns_fly_mntr_chnls(:,c);
lns_cnt_avg_SEM(:,cc)=filterf;
lns_cnt_avg_SEM(:,cc+)=SEM;
lns_cnt_avg_SEM(:,cc+)=length(Mdummy.data(:,::));
figure(); SEM=[];
cc+=;
end
save([‘lns_cnt_avg_SEM ’ ‘ ‘ char(LD) ’ ‘ ,’ ‘ lns_cnt_avg_SEM’ ];save([‘lns_cnt_avg_SEM_lbls’ ‘ ‘ _
char(LD)’ ‘ ,’ ‘ lns_cnt_avg_SEM’ ]);% to run damalyze of each flies to generate individual overall mean counts, period, ri, rs, phi, and rho

fly_vrs_lbl(:,1)=; fly_vrs=[];
while <length(lns_fly_mntr_chnls(:,::))
    Mdummy=[];
%damalyze_par = p.plotcol=[‘acto’ ‘flyf’ ‘auto’ ];
figure;
{table_v}=JA_loc_dam_analyze(fig_name,Mdummy,0:1:length(Mdummy.f(:,::)),p);
[phi1,ci,c2,rohi]=peakphase(Mdummy.f,Mdummy.int,1);
l2=length(table_v(:,::));
if ==
    fly_vrs(:,1:12,:)=table_v; fly_vrs(:,1:12,:)=phi1;
    fly_vrs(:,1:12,:)=rohi;
    fly_vrs_lbl(:,1:12,:)=lns_fly_mntr_chnls(:,c);
else
    fly_vrs(:,1:11+2,1:)=table_v;
    fly_vrs(:,1:11+2,1:)=phi1;
    fly_vrs(:,1:11+2,1:)=rohi;
    fly_vrs_lbl(:,1:11+2,1:)=length(fly_vrs_lbl(:,1:));length(table_v(:,::)),::)=lns_fly_mntr_chnls(:,c);
end
end

table_v=[]; phi=[]; rohi=[];
% adding night and day and ratio activity counts
idx_nt_actv=find(time_ZT(:,::)>12);
idx_dy_actv=find(time_ZT(:,::)<12);
idx_m_pk=find(time_ZT(:,::)>18 | time_ZT(:,::)<6);
idx_e_pk=find(time_ZT(:,::)<18 & time_ZT(:,::)>6);
num_nts=length(find(time_ZT(:,::)>12))/24;
num_bet=length(find(time_ZT(:,::)<18 & time_ZT(:,::)>6))/24;
num_m_pk=length(find(time_ZT(:,::)>18 | time_ZT(:,::)<6))/24;
num_e_pk=length(find(time_ZT(:,::)<18 & time_ZT(:,::)>6))/24;
if ==
    for =1:12
        fly_vrs(:,1,:)=mean(Mdummy.data(:,::));
        fly_vrs(:,1,:)=(sum(Mdummy.data(:,::))/num_fll_dys);
        fly_vrs(:,1,:)=(sum(Mdummy.data(idx_dy_actv,::))/num_dys);
        fly_vrs(:,1,:)=(sum(Mdummy.data(idx_nt_actv,::))/num_nts);
        fly_vrs(:,1,:)=(sum(Mdummy.data(idx_m_pk,::))/num_m_pk);
        fly_vrs(:,1,:)=(sum(Mdummy.data(idx_e_pk,::))/num_e_pk);
        fly_vrs(:,1,:)=(sum(Mdummy.data(:,::))/fly_vrs(:,1,:));
    end
else
    for =1:11+12
        fly_vrs(:,1,:)=mean(Mdummy.data(:,::));
        fly_vrs(:,1,:)=(sum(Mdummy.data(:,::))/num_fll_dys);
        fly_vrs(:,1,:)=(sum(Mdummy.data(idx_dy_actv,::))/num_dys);
        fly_vrs(:,1,:)=(sum(Mdummy.data(idx_m_pk,::))/num_m_pk);
        fly_vrs(:,1,:)=(sum(Mdummy.data(:,::))/fly_vrs(:,1,:));
    end
end
MATLAB Code

```matlab
% MATLAB Code

% Calculating line averages for variables
list=[]; list=unique(fly_vrs_lbl); list_cnt_Avg_SEM_lbls = {...}
for i=1:length(list)
    v=mean(list_cnt_Avg_SEM_lbls{i});
    list_cnt_Avg_SEM(i)=v;
end

% Error bars
for v=1:length(list_cnt_Avg_SEM_lbls)
    list_cnt_Avg_SEM_lbls(i,v)=std(list_cnt_Avg_SEM_lbls{i,v});
end

% Saving plots
save(fullfile('fly_vrs_lbl', '_char(LD)','fly_vrs_lbl'); save(fullfile('fly_vrs_lbl', '_char(LD)'),'fly_vrs_lbl');

% Creating p-values for overall means
idx_ctrl=find(strcmp(fly_vrs_lbl{i},CtrlLine));
for v=1:length(idx_ctrl)
    p=2*tcdf(abs(t),[n1 n2-2]);
    list_vrs_p=[list_vrs_p; 1, v];
end
save(fullfile('list_vrs_p', '_char(LD)'),'list_vrs_p');

% Peak circ plot

function JA_loc_plot_all_vrs(LD, CtrlLine, TrtLine, LinesCode, lns_fly_mntr_chnl, lns_vrs_lbl, lns_vrs_lbl, lns_vrs_SEM, lns_vrs_n, lns_vrs_p, time_ZT)
```

177
```
% JA_loc_plot_all_vrs('DD','LCSisoX','All',LinesCode,lns_fly_mntr_chnls,fly_vrs,fly_vrslbl,lns_cnt_Avg_SEM,lns_cnt_Avg_SEM_lbls,lns_vrs_Avg,lns_vrs_SEM,lns_vrs_n,lns_vrs_p,Time_TV)

% JA_loc_plot_all_vrs('LD','LCSisoX','All',LinesCode,lns_fly_mntr_chnls,fly_vrs,fly_vrslbl,lns_cnt_Avg_SEM,lns_cnt_Avg_SEM_lbls,lns_vrs_Avg,lns_vrs_SEM,lns_vrs_n,lns_vrs_p,Time_TV)

Letters=[ 'x'; 'x'; '1'; 'J'; 'K'; 'L'; 'M'; 'X'; 'H'; 'B'; 'C'; 'F'; 'D'; 'E'; 'G' ];
Lines=[];
if strcmp('TrtLine','All')=='
    Lines=TrtLine;
else
    Lines=unique(lns_cnt_Avg_SEM_lbls(1,:));
end
ctrl_avg_idx=[]; ctrl_sem_idx=[];
ctrl_avg_idx=min(find(strcmp(lns_cnt_Avg_SEM_lbls(:,1),CtrlLine)));
ctrl_sem_idx=max(find(strcmp(lns_cnt_Avg_SEM_lbls(:,2),CtrlLine))-1);
ctrl_idx=find(strcmp(lns_vrs_Avg(:,1),CtrlLine));
ctrl_n_idx=strrep(CtrlLine(2:end), '_','-');
for L=1:length(Lines)
    Line=Lines(L);
    if isempty(LinesCode(find(strcmp(LinesCode(:,1),Line),2)))
        LineDesc='None';
    else
        LineDesc=LinesCode(find(strcmp(LinesCode(:,1),Line),2));
    end
    LineDesc
    % Plotting CPS time series
    trt_avg_idx=[]; trt_sem_idx=[];
    trt_avg_idx=min(find(strcmp(lns_cnt_Avg_SEM_lbls(:,1),Line)));
    trt_sem_idx=max(find(strcmp(lns_cnt_Avg_SEM_lbls(:,2),Line))-1);
    trt_n_idx=find(strcmp(lns_vrs_Avg(:,1),Line));
    if cell2mat(strfind(Line,''))
        trt_n_idx=strrep(Line(2:end), '_','-');
    else
        trt_n_idx=Line(2:end);
    end
    grey = [0.4,0.4,0.4];
    figure;
    subplot(2,2,[11,14,23,36,45,58,67,80,89,102]);
    x=lns_cnt_Avg_SEM(:,1)-1;
    errorbar(x,lns_cnt_Avg_SEM(:,ctrl_avg_idx),lns_cnt_Avg_SEM(:,ctrl_sem_idx),'.-' ,'
    'LineWidth', 0.5, 'MarkerEdgeColor', 'none', 'MarkerFaceColor', 'k', 'MarkerSize', 0.5);
    xlim([0 length(lns_cnt_Avg_SEM(:,1))]);
    ZT0 = find((round(time_ZT(:,1))*10000) / 10000.0)==1);
    ZT12 = find((round(time_ZT(:,1))*10000) / 10000.0)==12);
    ZT_xcat = [ (ZT0, ZT12); ZT_x = sort(ZT_x);
    for m=length(ZT_x) if ZT_x(m)==ZT_x(z-1)+
        if find(min(time_ZT(ZT_x(z)),time_ZT(ZT_x(z-1)))) == 1;
            ZT_x(z)=NaN;
        else
            ZT_x(z-1)=NaN;
        end
    end
    ZT_x=ZT_x(~isnan(ZT_x));
    set(gca,'XTickLabel',ZT_x);
    set(gca,'TickLabel',floor(time_ZT(ZT_x,5)), 'FontSize', 1);
    title(trt_n_title ' LineDesc[I] ' 'FontSize', 6);
    set(gca,'FontSize', 5);
    hold all
    errorbar(x,lns_cnt_Avg_SEM(:,trt_avg_idx),lns_cnt_Avg_SEM(:,trt_sem_idx),'.-','
    'LineWidth',0.5, 'MarkerEdgeColor', 'none', 'MarkerFaceColor', 'grey', 'MarkerSize', 0.5)
    if strcmp(LD,'DD')
        xlabel('Time (CT)', 'FontSize', 7);
    else
        xlabel('Time (ZT)', 'FontSize', 7);
    end
    ylabel('Mean Counts/Min');
    if trt_n_title ' 
        num2str(lns_cnt_Avg_SEM(:,ctrl_n_idx)) ' '), [trt_nm ' 
        num2str(lns_cnt_Avg_SEM(:,trt_n_idx)) '); %24 because in the beginning, we don't have data from all exp
doing to alignment
    set(leg,'Location', 'Northeast', 'Orientation', 'horizontal', 'Box', 'off', 'FontSize', 7)
```

```matlab
% have data from all exp

% Rhythm Strength
num2

ylabel
xlim
set(gca,'XTickLabel',floor(time_ZT(ZT_x)), 'FontSize', 5);
hold all
plot(x,lns_cnt_Avg(SEM(:,ctrl_avg_idx)),'-s','color',grey,'LineWidth',0.5,'MarkerEdgeColor','none','MarkerFaceColor','k','MarkerSize',0.5)

xlabel('Time (CT)', 'FontSize', 7);

end

ylabel({{'Mean Counts/Min';' (No ErrorBars)'},'FontSize', 7});

leg = legend({{ctrl_nm ' {' numZstr(lns_cnt_Avg(SEM(:,ctrl_avg_idx)) ' ')},[trt_nm {' numZstr(lns_cnt_Avg(SEM(:,trt_n_idx)) ' ')}}; %24 because In the beginning, we don't have data from all exp due to alignment

set(leg,'Location','Northeast','Orientation','horizontal','Box', 'off','FontSize', 7)

uicontrol({'BackgroundColor','w','FontSize',5,'Style', 'text','String', ['K', 'Units','normalized','Position', [0.01 0.01 0.5 0.5] ]);

uicontrol({'BackgroundColor','w','FontSize',5,'Style', 'text','String', [ ctrl nm {' numZstr(TableFiles CPS(24,((LE8)-5)) )' }, 'Units','normalized', 'Position', [0.71 0.975 0.1 0.02] ];

var_col num_lbls='Line ' 'Mean Period (Autocorrelation) ' 'Mean Rhythmicity Index ' 'Mean Rhythm Strength ' 'Mean Phase ' 'Mean Phase Consistency ' 'Mean (Counts/min) ' 'Mean Counts/24hrs ' 'Mean (Day Counts) ' 'Mean (Night Counts) ' 'Mean (Night/Day Counts) ' 'Mean (M peak Counts) ' 'Mean (K peak Counts) ' 'Mean (E/M Counts) ';

% Plotting Bar graphs
for var_col=1,1:15
if var_col==1
xm = length(lns_cnt_Avg(SEM(:,1)));
plot(x,lns_cnt_Avg(SEM(:,ctrl_avg_idx)),'-ks','LineWidth',0.5,'MarkerEdgeColor','none','MarkerFaceColor','k','MarkerSize',0.5)
set(gca,'XTick',ZT_x);
set(gca,'XTickLabel',floor(time_ZT(ZT_x)), 'FontSize', 5);

hold all
plot(x,lns_cnt_Avg_SEM(:,trt_avg_idx),'-s','color',grey,'LineWidth',0.5,'MarkerEdgeColor','none','MarkerFaceColor',grey,'MarkerSize',0.5)
xlim([0 length(lns_cnt_Avg_SEM(:,1))]);
if strmp(LD,'DD')>
 xlabel('Time (CT)', 'FontSize', 7);
else
 xxlabel('Time (ZT)', 'FontSize', 7);

end
ylabel({{'Mean Counts/Min';' (No ErrorBars)'},'FontSize', 7});

leg = legend({{ctrl_nm ' {' numZstr(lns_cnt_Avg_SEM(:,ctrl_avg_idx)) ' ')},[trt_nm {' numZstr(lns_cnt_Avg_SEM(:,trt_n_idx)) ' ')}}; %24 because In the beginning, we don't have data from all exp due to alignment

set(leg,'Location','Northeast','Orientation','horizontal','Box', 'off','FontSize', 7)

```
trt_idx=find(strncmp(fly_vrs_lbl(:,1),CtrlLine));

idx_ctrl=find(strncmp(fly_vrs_lbl(:,1),CtrlLine));
idx_trt=find(strncmp(fly_vrs_lbl(:,1),Line));

subplot(2,2,[52:528,542:544,564:566,588:588,608:610]);
JA_multiple_test_nrmLz(fly_vrs(idx_ctl,5),fly_vrs(idx_trt,5),6,3,3,'k','grey',
title('All Flies','FontName', 'FontWeight', 'Bold');

JA_bivariate_test_nrmLz(fly_vrs(idx_ctl,5),fly_vrs(idx_trt,5),fly_vrs(idx_trt,5),fly_vrs(idx_ctl,5),3,3,'k ',
'grey',
title('All Flies','FontName', 'FontWeight', 'Bold');

saveas(gcf, ['fig'], 'fig'); saveas(gcf, ['Line'], 'fig'); saveas(gcf, ['char(LD) FIG'], 'fig'); saveas(gcf, ['char(LD) jpg'], 'jpg');

saveas(gcf, ['Line'] 'char(LD) eps', 'eps'); saveas(gcf, ['Line'] 'char(LD) pdf', 'pdf');

close all;
end
Specialized cells tag sexual and species identity in
*Drosophila melanogaster*

Jean-Christophe Billiter, Jade Atallah, Joshua J. Krupp, Jocelyn G. Millar & Joel D. Levine

Social interactions depend on individuals recognizing each other, and in this context many organisms use chemical signals to indicate species and sex[1]. Cuticular hydrocarbon signals are used by insects, including *Drosophila melanogaster*, to distinguish conspecific individuals from others[2]. These chemicals also contribute to intraspecific courtship and mating interactions[3-5]. However, the possibility that sex and species identification are linked by common chemical signalling mechanisms has not been formally tested. Here we provide direct evidence that a single compound is used to communicate female identity among *D. melanogaster* and to define a reproductive isolation barrier between *D. melanogaster* and sibling species. A transgenic manipulation eliminated cuticular hydrocarbons by ablating the oenocytes, specialized cells required for the expression of these chemical signals. The resulting oenocyte-less (oe-) females elicited the normal repertoire of courtship behaviours from males, but were actually preferred over wild-type females by courting males. In addition, wild-type males attempted to copulate with oe- males. Thus, flies lacking hydrocarbons are a sexual hypertimulus. Treatment of virgin females with the aversive male pheromone cis-vaccenyl acetate (cVA) significantly delayed mating of oe- females compared to wild-type females. This difference was eliminated when oe- females were treated with a blend of cVA and the female aphrodisiac (7Z,11Z)-heptacosadiene (7,11-HD), showing that female aphrodisiac compounds can attenuate the effects of male aversive pheromones. 7,11-HD also showed to have a crucial role in interspecific encounters. Specifically, the species barrier was lost because males of other *Drosophila* species courted oe- *D. melanogaster* females, and *D. simulans* males consistently mated with them. Treatment of oe- females with 7,11-HD restored the species barrier, showing that a single compound can confer species identity. These results identify a common mechanism for sexual and species recognition regulated by cuticular hydrocarbons.

*D. melanogaster* produces hydrocarbons of various chain lengths, including unbranched alkanes, methyl-branched alkanes, alkenes and derivatives thereof. The alkenes are expressed sex-specifically, and have been associated with both sex and species discrimination[6-9]. Compared to females, males express high levels of the nonanoaldehyde (2Z,7-tricosene (7-T)), which has been reported to increase females’ receptivity to mating attempts. Moreover, 7-T is repulsive to other males and may prevent male-male interactions[10]. In contrast, females produce sex-specific dienes such as (7Z,11Z)-heptacosadiene (7,11-HD) and (7Z,11Z)-nonacosadiene (7,11-ND), which act as aphrodisiac pheromones for *D. melanogaster* males[11,12]. Hydrocarbons are strongly associated with sexual recognition, because wild-type male court males that have been genetically modified to express female hydrocarbons, indicating that the mutants are perceived as females[13].

There are still large gaps in our knowledge of the functions of individual hydrocarbons and the tissues where these compounds are synthesized. As in other insects[14], specialized cells called oenocytes, located on the inner surface of the abdominal cuticle, are thought to be the site of hydrocarbon biosynthesis in *D. melanogaster*. Consistent with this hypothesis, desaturase 1 (desat1), which encodes an enzyme involved in hydrocarbon synthesis[15], is expressed in *Drosophila* oenocytes[16] (Fig. 1a). Previous studies have demonstrated that genetic feminization of these cells results in production of female hydrocarbons by male flies; however, these and other manipulations have been confounded by the concurrent feminization of cells in many other sexually dimorphic tissues, including the central nervous system[17]. To test the hypothesis that these cells are required for production of chemical signals used in sexual and species recognition, we used the Gal4-UAS system[18] to target transgene expression specifically to the adult oenocytes. We generated an oenocyte Gal4 driver (Fig. 1b) derived from the regulatory sequence of one of the desat1 promoters[19] that is expressed specifically in oenocytes of adult females (Fig. 1a–c). The driver is also expressed in the larval oenocytes and in the reproductive organs of adult males (Fig. 1a, c and Supplementary Fig. 1). We used this driver to ablate adult oenocytes by inducing expression of the pro-apoptotic gene head involution defective (hid) also called Wrinkled[20]. This approach initially caused lethality in larvae, probably due to the destruction of the larval oenocytes. To circumvent this problem we blocked the driver’s action during development using the Tubulin-Gal80[21] transgene. Using this method, we generated adult flies without oenocytes (oe-) (Fig. 1d, e). Analysis of whole-body hydrocarbon extracts confirmed that both oe- males and females were essentially devoid of these compounds (Fig. 1f, g and Supplementary Tables 1 and 2 for quantification), showing that the oenocytes are necessary for hydrocarbon display in *D. melanogaster*. The male pheromone cis-vaccenyl acetate (cVA) was unaffected in oe- males (Fig. 1g, g) because this compound is synthesized in the ejaculatory bulb[22]. The oe- transgenic strain therefore provided a ‘blank slate’ for evaluating the role of hydrocarbons in intra- and interspecific communication.

We assayed sexual behaviour of oe- flies to test hydrocarbon function during reproduction. Despite the association of hydrocarbon signals and *Drosophila* courtship, absence of these signals did not alter courtship behaviours per se. The oe- males displayed normal courtship behaviour towards wild-type females, but slightly less intense than control males (Fig. 2a and Supplementary Table 3). However, wild-type females were less receptive to oe- males than control males, with oe- males taking almost four times as long to achieve mating (Fig. 2a). Thus, hydrocarbons of males do not seem to affect their own courtship behaviour, but rather, influence the receptivity of females to their mating attempts. However, we cannot exclude the influence of non-oenocyte cells within the male reproductive organs that may have been affected by the ablation. Notably, oe- males elicited courtship and copulation attempts from both...
One, Two, and Many—A Perspective on What Groups of *Drosophila melanogaster* Can Tell Us About Social Dynamics

Jonathan Schneider, Jade Atallah, and Joel D. Levine

Department of Biology, University of Toronto at Mississauga, Mississauga, Ontario, Canada

I. Introduction
   A. Caveat

II. The Behavioral Effects of Social Context
   A. Social learning and memory
   B. Social synchronizing of activity and rest
   C. Aggression
   D. Mating, paternity, and offspring

III. System-Level and Network Approaches to Social Context

IV. Conclusion

References

Abstract

In the natural world, interactions between individuals occur in groups: an individual must recognize others, identify social opportunities, and discriminate among these options to engage in an interactive behavior. The presence of the group is known to exert an influence on individual group members, and this influence may feed back through the individual to affect behavior across the group. Such feedback has been observed in *Drosophila melanogaster*, for example, when mating frequency increases in groups composed of mixed strains compared to homogenous groups (Krupp et al., 2008 and Billette et al., 2012). A working hypothesis is that social processes—to recognize, identify, discriminate, and engage—are innate. They rely on a combination of genetic inheritance,
Regulation of onset of female mating and sex pheromone production by juvenile hormone in Drosophila melanogaster

Julide Bilen1,2, Jade Atallah1,2, Reza Azandish1,2, Joel D. Levine1, and Lynn M. Riddiford3

1Janelia Farm Research Campus, Howard Hughes Medical Institute, Ashburn, VA 20147; and Departments of 1Biology and 2Cell and Systems Biology, University of Toronto Mississauga, Mississauga, ON, Canada L5L 1C6

Contribution by Lynn M. Riddiford, September 25, 2013 (sent for review May 12, 2013)

Juvenile hormone (JH) coordinates timing of female reproductive maturation in most insects. In Drosophila melanogaster, JH plays roles in both mating and egg maturation. However, very little is known about the molecular pathways associated with mating. Our behavioral analysis of females genetically lacking the corpora allata, glands that produce JH, showed that they were courted less by males and mated later than control females. Application of the JH mimic, methoprene, to the allatotropin females just after eclosion rescued both the male courtship and the mating delay. Our studies of the null mutants of the JH receptors, Methoprene-tolerant (Mrt) and germ cell-expressed (gec), showed that lack of Mrt in Mrt female delayed the onset of mating, whereas lack of Gec had little effect. The Mrt females were shown to be more attractive but less behaviorally receptive to copulation attempts. The behavioral but not the attractiveness phenotype was rescued by the Mrt genomic transgene. Analysis of the female cuticular hydrocarbon profiles showed that corpora allata ablation caused a delay in production of the major female-specific sex pheromones (the 7,11-C27 and -C29 dienes) and a change in the cuticular hydrocarbon blend in the Mrt null mutant, by 48 h, the major C27 diene was greatly increased relative to wild type. In contrast, the gec null mutant females were courted similarly to control females despite changes in certain cuticular hydrocarbons. Our findings indicate that JH acts primarily via Mrt to modulate the timing of onset of female sex pheromone production and mating.

All aspects of reproductive maturation in animals including gonad development, pheromone production for communication between the sexes, and mating behavior need to be precisely timed and coordinated to ensure reproductive success, and regulatory hormones are the key to this timing and coordination (1, 2). In most female insects, the sessutermporal juvenile hormone (JH) regulates and coordinates reproductive maturation of the ovaries (3) and often sex pheromone synthesis and mating behavior (4, 5). In Drosophila melanogaster, females are unresponsive to male courtship attempts just after eclosion, but by day 3, most become receptive and mate (6, 7). Implantation of JH-secreting corpora allata (CA) (6) just before eclosion or application of methoprene (8), a JH mimic (JHM), caused females to mate precociously. Decreased JH in the agouti mutant (9) or after treatment of females with 20,26-progesterone (a compound agonistic for the CA (10)), reduced female mating. These findings suggest that JH may play a role in the maturation of female receptivity in D. melanogaster.

The switch from an unresponsive to a receptive state requires coordination of neural activity critical for female mating behaviors with production of sex pheromones that attract the male (11–13). The Drosophila sex pheromones are a subgroup of the cuticular hydrocarbons (CHC) that mediate chemical communication for both sex and species recognition (see refs. 14 and 15 for reviews). In D. melanogaster, two CHCs, (Z,2)-7,11-C27:2 (C27) and (Z,4)-7,11-C29:2 (C29) dienes, are female-specific; these dienes are known to play roles in male choice preference and the onset of male courtship (15–18). Overexpression of the JH esterase-binding protein DmPSP after eclosion, which is thought to reduce the JH titer, caused a reduction in the synthesis of these female-specific dienes (19). The role of JH in the biochemistry of these CHCs is unknown.

In D. melanogaster, there are two basic helix-loop-helix proteins encoded by the paralogous genes, Methoprene-tolerant (Mrt) and germ cell expressed (gec), which act as JH receptors in both metamorphosis and reproductive maturation (20–23). Loss of function of both receptor genes is necessary to mimic the effect of genetic ablation of the CA in larvae, which causes prepupal lethality (23–25). In the adult, loss of Mrt causes both delayed and reduced fecundity, whereas loss of gec has only a slight effect on ovarian maturation (25). To elucidate the molecular mechanisms underlying JH’s role in the onset of mating in D. melanogaster females, we have genetically ablated the CA in the developing adult and found that the onset of mating behavior was delayed, and this delay could be rescued by JH. This action of JH was dependent only on the Mrt receptor and was partly due to modulation of sex pheromone production.

Results

Genetic Ablation of the CA in the Developing Females Delays the Onset of Mating. To address the role of JH in female mating behavior, we genetically ablated the CA in developing Drosophila females by targeting the expression of diphtheria toxin (DTT) to the CA via the CA-specific 4in2p-galactosidase (GAL4) driver (Fig. 1 A–C). Because the removal of the CA is prepupal lethal (23, 24), we used the temperature-sensitive GAL80 (GAL80ts) transgene to bypass this critical period for JH during development. At 60 h after puparium formation (APF), we shifted the popu...
Appendix V  PERMISSIONS FOR REPRODUCING PUBLISHED MANUSCRIPTS

A.V.1  PERMISSION FOR REPRODUCING (SCHNEIDER ET AL., 2012A)

Licensee: Jade Atallah
License Date: Nov 19, 2014
License Number: 3512531400016
Publication: Elsevier Books
Title: Advances in Genetics
Type Of Use: reuse in a thesis/dissertation
Total: 0.00 USD
A.V.2 Permission for Reproducing (Billeter et al., 2009)

Permission requests

On this page
Nature Publishing Group grants permission for authors, readers and third parties to reproduce material from its journals and online products as part of another publication or entity. This includes, for example, the use of a figure in a presentation, the posting of an abstract on a web site, or the reproduction of a full article within another journal. Certain permissions can be granted free of charge; others incur a fee.

For answers to frequently asked questions click here

Types of permission request

Permission can be obtained for re-use of portions of material - ranging from a single figure to a whole paper - in books, journals/magazines, newsletters, theses/dissertations, classroom materials/academic course packs, academic conference materials, training materials (including continuing medical education), promotional materials, and web sites. Some permission requests can be granted free of charge, others carry a fee.

Nature Publishing Group rarely grants free permission for PDFs of full papers to be reproduced online, however e-print PDFs can be purchased as commercial reprints. If you wish to purchase multiple stand-alone copies of a Nature Publishing Group paper, which is then printed and shipped to you, please go to commercial reprints.

Permission request options

Permission requests from authors
The authors of articles published by Nature Publishing Group, or the authors' designated agents, do not usually need to seek permission for re-use of their material as long as the journal is credited with initial publication. For further information about the terms of re-use for authors please see below.

Author Requests

If you are the author of this content (or his/her designated agent) please read the following. Since 2003, ownership of copyright in original research articles remains with the Authors*, and provided that, when reproducing the Contribution or extracts from it, the Authors acknowledge first and reference publication in the Journal, the Authors retain the following non-exclusive rights:

a. To reproduce the Contribution in whole or in part in any printed volume (book or thesis) of which they are the author(s).

b. They and any academic institution where they work at the time may reproduce the Contribution for the purpose of course teaching.

c. To reuse figures or tables created by them and contained in the Contribution in other works created by them.

d. To post a copy of the Contribution as accepted for publication after peer review (in Word or Tex format) on the Author's own web site, or the Author's institutional repository, or the Author's funding body's archive, six months after publication of the printed or online edition of the Journal, provided that they also link to the Journal article on NPG's web site (eg through the DOI).

NPG encourages the self-archiving of the accepted version of your manuscript in your funding agency's or institution's repository, six months after publication. This policy complements the recently announced policies of the US National Institutes of Health, Wellcome Trust and other research funding bodies around the world. NPG recognizes the efforts of funding bodies to increase access to the research they fund, and we strongly encourage authors to participate in such efforts.

Authors wishing to use the published version of their article for promotional use or on a web site must request in the normal way.

If you require further assistance please read NPG's online author reuse guidelines.

http://www.nature.com/reprints/permission-requests.html
Note: British Journal of Cancer maintains copyright policies of its own that are different from the general NPG policies. Please consult this journal to learn more.

* Commissioned material is still subject to copyright transfer conditions

Ordering permissions online
Most permission requests can be granted online through the Rightslink® service. To use this system, locate the article for which you wish to request a permission by using the advanced search.

Beneath the listing for the paper (when found through the search), you will find a clickable option 'Rights and Permissions', which will take you to the order entry page from which you can request your permission.

To request a permission through the Rightslink® service you will be required to create an account by filling out a simple online form. Most customers can order and, where necessary, make payment - by credit card or invoice - through the Rightslink® service. Rightslink then issues a printable licence, which is the official confirmation that permission has been granted.

For questions about Rightslink® accounts, please telephone Copyright Clearance Center customer support on 877 622 3543 (toll free inside the USA) or e-mail customerscare@copyright.com. Note that once you have created a Rightslink® account you can use this in the future to request and pay for permissions from other participating publishers.

If the abstract or article does not contain a 'Rights and permissions' link, or the article that you wish to request permission to re-use has not yet been published, or pre-dates our online archive, please e-mail the appropriate permissions manager for the journal.