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The Effects of Serotonin on the Courtship Behavior of Drosophilia melanogaster

Nicholas James Brandmeir

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THE EFFECTS OF SEROTONIN ON THE COURTSHIP BEHAVIOR OF

DROSOPHILA MELANOGASTER

By

Nicholas James Brandmeir

BA Cornell University, 2002

A THESIS

Submitted in partial fulfillment of the

Requirements for the Degree of

Master of Science

(in Zoology)

The Graduate School

The University of Maine

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In *Drosophila*, the male courtship ritual is stereotyped and under the control of several genes including *fruitless*. In previous studies, it has been shown that various *fruitless* alleles cause phenotypic abnormalities in the *D. melanogaster* courtship ritual. It is also known that there is a high level of co-expression of both the *fruitless* gene product and serotonin in specific neurons of the *Drosophila* CNS. This study examines the role of serotonin in the *Drosophila* male courtship ritual by using a mutant strain, *Ddc*[^1], in which the production of serotonin is blocked above 30°C. In this study an increase in temperature caused a decrease in general activity as well as a decrease in several courtship behaviors. Also above 30°C there was increased homosexual courtship among males. *Ddc*[^1] flies were observed copulating with a smaller latency than wild-type flies. Finally, deprivation of serotonin decreased the frequency of wing extensions performed by a courting male.
ACKNOWLEDGEMENTS

I would like to thank the members of my committee for their excellent advice and support throughout this research. I would also like to thank Dr. Irv Kornfield for providing the video equipment necessary to complete this work. Also invaluable was Allison Cox, who taught me the ins and outs of fly husbandry and courtship. I wish also to thank Benjamin Hysell for his help with programming in visual basic. Finally, I wish to thank my wife and family for their support during this research.
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Introduction

Mating

Prior to copulation the mating sequence of *Drosophila melanogaster* features a period of courtship (Spieth 1952). The courtship of *Drosophila* is stereotyped and composed of discrete steps all of which have been shown to be under some degree of genetic control (for a review of mating behaviors and their genetic control see (Yamamoto and others 1997), or (Hall 1994)) (Fig 1). Typically courtship begins with the male orienting toward the posterior of the female and following her. While oriented, the male will signal to the female, by extending and vibrating a wing, producing a mating song. Usually after singing, the male will ‘lick’ the female genitalia. Concurrent with these acts the male will tap the female with his forelegs. After these displays the female will accept or reject the male. If accepted by the female the male will mount the female and copulate, injecting sperm into the female. Post copulation, the male will disconnect genitalia from the female and dismount. It is common for males to make several unsuccessful attempts at copulation during a courtship.

Genes that affect courtship

As noted above there are many genes that affect the courtship of *Drosophila*. Many of these genes are involved in general neurological development or structure and have their effects through an enfeeblement of the organism, *yellow* for example (Bastock 1956). Still other mutants may show abnormal courtship due to deficits in sensory
apparatuses critical to successful courtship and mating. Examples of this would be the white mutation which causes deficits in a male's ability to track females (Sturtevant 1915) and the smellblind allele of the paralytic gene which causes slight decrements in male courtship and aberrant female response to male courtship (Tomkins and others 1980). Mutations controlling rhythms or other biological oscillators have also been shown to affect courtship, the classical case being period which has a large effect on the qualities of the male courtship song (Alt and others 1998). Further, various learning mutations have been shown disrupt some normal courtship-related behavior. Male flies must learn not to court recently mated females through interacting and realizing that the female will block the vast majority of attempted copulations (Gailey and others 1991). The gene Ddc which encodes dopa decarboxylase, an enzyme that is necessary for the synthesis of the neurotransmitters dopamine and serotonin, has been shown to abrogate learned mating behaviors as well as other learned behaviors (Tempel and others 1984). The final class of genes that have an effect on courtship behavior are genes acting in the sex determination hierarchy. These genes are responsible for determining and directing
the sexual dimorphisms present in *Drosophila* (Taylor and others 1994). This particular class of mutation will be examined in greater depth below.

**The sex determination hierarchy**

In *Drosophila*, sex is determined by genes whose hierarchical expression produces sexually dimorphic phenotypes (for a review see (Cline and Meyer 1996)). In normal flies the hierarchy is controlled by the ratio of X chromosomes to autosomes. In XX transcription factors activate the embryonic expression of *Sex-lethal (Sxl)*. This expression of Sxl protein, SXL\textsubscript{PC}, acts as a splicing factor on later expression of Sxl. It functions by splicing out an intron containing early stop codons from the middle of the transcript. This new Sxl protein, SXL\textsubscript{PM}, will, in turn, regulate the splicing of pre-mRNA from the *transformer* gene. *transformer* and the gene *transformer-2* together regulate the splicing of the gene *doublesex*. This splice pattern leads to production of the female-specific DSX protein product. DSX acts as a transcription factor, activating genes necessary for the development of female specific morphology such as external genitalia and particular areas of the CNS such as abdominal neuroblasts. In the male the chromosomal condition XY is normal, although X0 will also result in male phenotypes. In this condition, no SXL\textsubscript{PC} is produced and without its presence, male transcripts for SXL\textsubscript{PM} are not translated due to the continuing presence of early stop codons. Without SXL\textsubscript{PM} the male splices *tra* so that an early stop codon is contained within the mRNA and TRA never produced. This allows the default splicing of *doublesex* mRNA, which now acts as a male-specific transcription factor that controls the development of male-specific morphologies such as external genitalia and dimorphic mushroom bodies.
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<th>Gene</th>
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<td>Lawrence</td>
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<td>Behavioral sterility, male-male courtship, lack of male-specific muscle of</td>
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<td>or young males after initial introduction to females, previously mated females,</td>
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<td>Abnormal male courtship</td>
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<td>Dopa-transportase</td>
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The sex determination pathway is not a linear heirarchy but contains at least two significant branches (Ryner and others 1996). One branch, discussed above, is the \textit{doublesex} branch. It is chiefly responsible for controlling and directing the development of sexually dimorphic external structures like claspers or sex-combs. Another, the focus of this introduction is the \textit{fruitless} branch. The existence of the \textit{fru} branch was inferred from the observation that the male-specific Muscle of Lawrence depends upon the TRA protein but does not depend upon the DSX protein moreover \textit{doublesex} null mutants are largely normal in their mating behavior. This branch, though not as well characterized as the \textit{doublesex} branch, is known to control sex-specific mating behavior and the development of the Muscle of Lawrence.

\textit{fruitless}

\textit{fruitless} is a mutation on chromosome arm 3R of the \textit{Drosophila melanogaster} genome. It was isolated by Kulbir Gill in a mutagenesis experiment searching for sterile mutants (Gill 1963). Gill observed that \textit{fruitless} males mate abnormally, are behaviorally sterile, and court other males, often forming long courtship chains where each male both courts and is courted. Through deficiency mapping \textit{fruitless} was found to be located in segment 91B of chromosome (Gailey and Hall 1989).

\textit{fruitless} is a complex gene with many different alleles (Fig. 3). It has 4 separate promoters, P1-P4, with P1 being the most 5'. The promoters are the basis for some sexually dimorphic expression of \textit{fruitless}. The products formed by the P1 promoter are sexually dimorphic and subject to different splicing depending on the sex of the cell.
The products using the P2, P3 and P4 as 5' promoters; however, are expressed in both sexes. The transcripts of both the male-specific (FRU\textsuperscript{M}) and female-specific products contain a BTB domain, which is a common domain in dimerizing proteins. The final interesting characteristic of the fruitless locus is the presence of three separate Zn-finger domains at the 3' end. The Zn-finger domain is a well known domain that is known to interact with the structure of DNA in other transcription factors. Any of the three domains may be spliced into the mRNA product of...
the gene. These alternate splice variants have been shown to responsible for the sexually
dimorphic expression of fruitless (Demir and Dickson 2005). This work was by
observing the behavior of lines of flies that made only the male or female splice variant
regardless of sex. The presence of FRU\textsuperscript{M} in females was sufficient to cause done male
mating behavior. The presence of the BTB and Zn-finger domains strongly suggests

Figure 3: Map of the fruitless locus –
(A) Shows a high resolution map of the fruitless locus. Thick vertical lines represent
elements of interest in the sequence of the gene. P1-P4 are promoters that may be used to
generate different transcripts. A, B, C are Zn-finger domains that may be spliced in or
out to create splice variants. S and C1-C5 represent other exons. Blank triangles show
the insertion point of the P-element transposon used to create the allele. The arrow and
vertical dashed lines represent the location of breakpoints used to create the appropriate
allele. Thick horizontal lines represent various deficiencies (deletions) responsible for
creating the indicated allele. (B) Shows the exons present in the splice variants generated
from fruitless. (adapted from (Anand and others 2001))
that fruitless encodes a product that dimerizes to form a transcription factor (Ryner and others 1996) (for a list of relevant fruitless alleles see table 2).

Since Gill’s discovery of the first mutant fruitless allele there have been many more generated and investigated ((Gailey and Hall 1989); (Ryner and others 1996); (Ito and others 1996); (Villella and others 1997)). By examining the various phenotypes generated by these alleles, researchers have shed light on a large number of anatomical and physiological features under fruitless control.

Later work quantifying Gill’s initial observations of fru males found them to court females at a normal rate, but the courtship was less vigorous and effective than in wild-type (Hall 1978). In addition, the males were rendered completely sterile owing to the fact that they no longer attempted to copulate with females (Hall 1978). Further, Hall found that fru males were deficient in their rejection of courtship from other males and what’s more were able to stimulate courtship behavior in other males even when etherized or dismembered (Hall 1978). fru/fru court other fruitless males as well as other wild-type males. A remarkable manifestation of this homosexual courtship is the tendency of groups of males expressing abnormal fruitless product to form courtship chains. In these chains a male will be both courting a male in front of him and being

<table>
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<td>Chromosomal break</td>
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<td>(Gill 1963)</td>
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<tr>
<td>fruitless (fru')</td>
<td>P-element insertion</td>
<td>Between P3 and P4</td>
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</tr>
<tr>
<td>fruitless (fru')</td>
<td>P-element insertion</td>
<td>Just downstream of P2</td>
<td>(Villella and others 1997)</td>
</tr>
<tr>
<td>fruitless (fru')</td>
<td>P-element insertion</td>
<td>Just upstream of P3</td>
<td>(Villella and others 1997)</td>
</tr>
<tr>
<td>fruitless (fru')</td>
<td>P-element insertion</td>
<td>Just upstream of P3</td>
<td>(Ito and others 1996)</td>
</tr>
</tbody>
</table>

Table 2: Brief List of Phenotypically Important fruitless alleles
courted by a male to his posterior. Other alleles of the fru locus were shown to induce male-male courtship and chaining behaviors including fru² (Gailey and Hall 1989), fru³, and fru⁴ (Villella and others 1997) (Fig. 4).

As mentioned above, the male courtship sequence involves ‘singing’, where the male will extend one wing and vibrate it, producing a courtship song. It was noted during early investigations that fru¹ mutants will sometimes extend both wings simultaneously, a behavior never seen in wild-type flies (Hall 1978). The Drosophila courtship song contains two distinct elements: sine song and pulse song. Villella et al. measured the song characteristics of various fru alleles, it was found

![Figure 4: Chaining in fruitless –
Shows males possessing various mutant genotypes of fruitless forming courtship chains.
(Adapted from (Villella and others 1997))](image)

that fru¹ and fru² mutants sang abnormally while mutants expressing the fru³ or fru⁴
genotypes failed to sing at all (Villella and others 1997). This work showed that *fruitless* plays some role in not only directing the courtship, but also in the qualities of the particular courtship steps.

*fruitless* expression is necessary for the development of the male-specific Muscle of Lawrence (Gailey and others 1991) (Fig. 5). This discovery was made by using both *fruitless* mutations and overlapping chromosomal deletions that encompassed the *fruitless* locus. In all cases of disrupted *fru* expression the Muscle of Lawrence was either missing or abnormal.

![Figure 5: Muscle of Lawrence – (A) Presence of the Muscle of Lawrence (arrowheads) in a normal male fly. (B) Male fly with Df(3R)ChaMi'/Df(3R)P14 (deletions that overlap *fruitless*) showing no Muscle of Lawrence. (adapted from (Gailey and others 1991)).](image)

For a time it was suspected that the Muscle of Lawrence is responsible for bending the male’s abdomen to copulate. This was shown not to be the case when it was discovered that *fru*/*fru* males are capable of mating and are fertile (Gailey and Hall 1989) even though they lack a normal Muscle of Lawrence (Gailey and others 1991).
This fact, combined with the observation that various fruitless mutants have extremely long mating durations compared to wild-type flies, led Lee to hypothesize that the Muscle of Lawrence is involved in unbending the male abdomen after mating. This hypothesis is supported by the dorsal location of the Muscle of Lawrence, which would indicate that its contraction would extend the abdomen (Lawrence and Johnston 1984).

As a mediator of behavior, fruitless must act on the nervous system of Drosophila in specific ways. Furthermore, fruitless's role in the Muscle of Lawrence also supported expression in the nervous system since development of the muscle is neuron-dependent (Lawrence and Johnston 1986). Areas of the Drosophila CNS had been previously implicated in controlling sex-specific courtship behaviors through the creation of sex-mosaics (Hall 1977). These sex-mosaics are flies possessing some male tissue and female tissue in different areas of the body. Male and female tissues are different morphologically and are able to be scored with high accuracy. Thus, if a certain area of the CNS, when feminized, eliminates male courtship behavior, it would be implicated in controlling courtship. The creation of markers specific for the male fruitless protein (FRU\textsuperscript{M}) made it possible to examine the expression patterns of fruitless in the Drosophila CNS directly through in situ hybridization (Lee and others 2000). These experiments showed that FRU\textsuperscript{M} is not present at all in the female. Also, this finding localized the expression of FRU\textsuperscript{M} to specific areas of the male CNS previously identified as being centers for courtship control (Ferveur and Greenspan 1998). mRNAs common to both males and females were previously shown to be transcribed from the P3 and P4 promoters and to be necessary for development (Ryner and others 1996).
These transcripts were not limited to neural expression patterns and were in fact found to be expressed in several tissues in the developing and adult fly (Lee and others 2000).

With FRUM localized to a select subset of neurons, it was possible to begin examining the character of the neurons involved with male courtship directly. Chief among the defining properties of a neuron is the neurotransmitter it produces. A given neuron produces only one type of neurotransmitter. The effect of that neurotransmitter is completely dependent on the receptors of the post-synaptic neuron (For example, in humans acetylcholine causes skeletal muscle to contract with a higher frequency but causes cardiac muscle to slow down.). By double labeling it is possible to search for co-expression of both a given neurotransmitter and fruitless. Lee and Hall performed such experiments by looking for the presence of serotonin (5HT) in cells expressing fruitless. Their results showed that serotonin was not expressed in the brain or thoracic segments of the male Drosophila ventral nerve cord (Lee and Hall 2001). However, serotonin was co-localized with fruitless expression in a set of sexually dimorphic neurons in the abdominal segments (s-Abg) (Lee and Hall 2001). Further, these serotonergic neurons were absent or not producing serotonin when the flies were expressing a mutant genotype of fruitless (Lee and Hall 2001). These neurons terminate directly on several male-specific reproductive structures including the testicular ducts, seminal vesicles, accessory glands, and the anterior ejaculatory duct (Lee and Hall 2001). These were the only neurons observed innervating these structures and the wild-type pattern and presence of these neurons was disrupted by the expression of different fruitless alleles associated with various courtship abnormalities (Lee and Hall 2001).
Knowing that *fruitless* controls the development of specific neurons that express a specific neurotransmitter still does not tell us how *fruitless* acts. Sexual dimorphism may be either the anatomical or the physiological. In this case, it must be determined whether *fruitless* is responsible for changing the developmental fate of neurons or changing their connectivity (anatomy) or if it instead alters the neurotransmitters or receptors produced by neurons (physiology). Much progress has been made in this area through the use of the GAL4-UAS reporter system.

By using this system, researchers found that the clusters of cells producing the reporter gene to be markedly similar in both the male and female CNS (Demir and Dickson 2005). This result supported the idea that nearly identical anatomies exist in both the male and female CNS and that the male-specific features caused by *fruitless* expression were due to changes in the physiology of the subset of CNS neurons. The striking anatomical dimorphisms that were found were located in the PNS, specifically in the sensory neurons of the olfactory, gustatory, and visual systems (Demir and Dickson 2005). Males possessed many more sensory neurons expressing FRUM than did females. The role of the olfactory neurons was tested through several clever experiments and shown to be involved in mate recognition in *Drosophila*. Somewhat ancillary to their original goal, Demir and Dickson seem to have also discovered the first set of sensory neurons in *Drosophila* known to respond to volatile female pheromones (Demir and Dickson 2005). From this study it seemed that *fruitless* mainly affected physiology.

It was demonstrated by other researchers that FRUM supports the development of male-specific CNS anatomy including differences in the number, projection pattern, and dendritic fields of specific neurons (Kimura and others 2005). These neurons together
formed a male-specific neuronal circuit, but in females these neurons undergo apoptosis. Thus, it seems clear that there are both anatomical and physiological processes under the control of *fruitless* that are important for male courtship behavior.

This section has been chiefly concerned with the actions of *fruitless* and how it controls behavior. It would be hard to overestimate the importance of this gene in our ability to understand *Drosophila* courtship, but even so we must remember that *fruitless* acts in a genetic pathway, much of which has yet to be elucidated. A tantalizing clue to a possible downstream gene was discovered with the observation of co-expression of serotonin and FRU\textsuperscript{M} in male-specific neurons. A logical follow up experiment to this discovery would be to investigate the role of the neurotransmitter serotonin itself in the courtship behavior of *Drosophila*.

**Ddc as a tool for analyzing serotonin**

Dopa decarboxylase is an important enzyme in the synthesis of serotonin from tryptophan (Livingstone and Tempel 1983) (Fig 6). The *Ddc* gene was first discovered by Wright in 1976, who isolated several mutant alleles of *Ddc* (Wright and others 1976). One such allele was *Ddc\textsuperscript{al*}, which allows normal production of dopa decarboxylase (DDC) and thus serotonin below the permissive temperature (24°C) but when reared above the restrictive temperature (29°C) DDC is rendered inactive through a conformational change and serotonin is no longer produced (Livingstone and Tempel 1983). Comparing a *Ddc\textsuperscript{rt*} fly kept at restrictive temperature with a wild type kept at the same temperature and with a *Ddc\textsuperscript{al*} kept at permissive temperature allows a researcher to measure precisely the effects of serotonin on any dependent variable.
As mentioned previously, this system has been used in investigating the effect of serotonin on learning in *Drosophila*. It was found that without serotonin, male flies fail to learn from any experience, including those involved with mating (Tempel and others 1984). Further Tempel reported no mating abnormalities due to a lack of serotonin. Despite this, the issue should be reexamined in light of the research cited above and other factors. The first issue that needs clarification is that Tempel was not chiefly concerned with mating behavior and so did not assay the steps of courtship individually (Tempel and others 1984). Secondly, a more recent, anecdotal report suggests that *Ddc*<sup>111</sup> flies have a much lower mating success rate when reared at a restricted temperature when compared to flies reared at a permissive temperature (Yamamoto and others 1997). These factors taken together give a clear imperative for a new, targeted investigation into the effects of serotonin on courtship.

**Figure 6: The production of serotonin by *Ddc***
Materials and Methods

Fly Culture

Flies were cultured on cornmeal-molasses-malt-yeast medium in 25 ml vials under uncrowded conditions at 23°C. Propionic acid was added to prevent mold infestation.

Fly Stocks

Canton-S (CS) flies were used as wild-type. Dopa-decarboxylase\textsuperscript{szl} (Ddc\textsuperscript{szl}) (Bloomington/3188) were used as serotonin-lacking flies due to the flies' mutant Dopa-decarboxylase\textsuperscript{szl} gene (Ddc\textsuperscript{szl}) gene which results in an inability to produce serotonin when reared above 29°C (Wright, 1981). A schematic of the treatments for each group of flies is provided in Figure 7.

General Activity

Before assaying mutant strains for abnormalities in any active behavior it is necessary to gauge the strains' general activity levels. This is to control for the possibility that a decrement in overall activity could also manifest itself as a decrement in behavioral vigor.

Five days before testing, 1-3 day old flies were gathered from their vials and segregated into new vials by sex and stock (i.e., CS males would go in one vial, Ddc females in another). The vials were then stored at either 23 or 30°C. After 5 days the flies were removed from storage and allowed to acclimate to room temperature for 2-4 hours after which the observations described below were made.
A single fly was aspirated into a clean, circular Plexiglas chamber 10 cm in diameter. The chamber was placed onto a black and white surface with gridlines forming 1-cm squares. The fly was allowed to acclimate for ~1 minute. The fly was then observed for 2 minutes and every gridline crossing was tallied. The chamber was washed in warm water and dried thoroughly between trials. The sample sizes used were: CS(23°C)=18, CS(30°C)=42, Ddc(23°C)=12, Ddc(30°C)=29.

**Mating Behavior**

24h prior to the first collection from a vial, the vial was cleared of all adults. The vial was then used as a source for virgin females and male flies every day for 10 consecutive days. Virgin females were collected within 24h of eclosion and stored together in a vial. A new vial was used for each collection. Males were collected and stored together in a vial for at least 5 days before being used as subjects. This was done to ensure that no male subjects were newly eclosed and to give enough time for the restrictive temperature to have an effect on the sensitive flies. After collection all flies were kept in a DD cycle at 23°C for females and either 23°C or 30°C for males.

Flies were tested through the use of a 10-chamber mating wheel. Each chamber was 8 mm tall and with a diameter of 8 mm (Ringo and others 1992). In each experiment up to 3 chambers of the wheel were filled. The flies were allowed to acclimate to the chamber environment for ~1 minute. After the acclimation period the two flies in each chamber were introduced to each other simultaneously by aligning the chambers in the mating wheel. The interactions of each mating pair were then monitored simultaneously.
for 40 minutes via video recording. Just as above, any result caused by both temperature and $Ddc$ is the result of a lack of serotonin.

The male flies serving as subjects were taken from either the CS or $Ddc^{es}$ stock. They were then reared at either 23° or 30°C. Then the males were introduced to a female in a mating wheel as described above and observed. Measurements were taken at the start of courtship and mating, and the termination of mating. Each time a male fly extended a wing, licked the female, or attempted copulation it was recorded. The sample sizes used were: CS(23°)=26, CS(30°)=35, Ddc(23°)=18, Ddc(30°)=25. For some analyses, subsets of data were used with the sample size used indicated.

**Homosexual Courtship**

The subject males were collected and reared exactly as described above. The target males were collected and reared identically to the subject males but were drawn only from the CS stock and reared only at 23°C. The subject male and target male were introduced through use of a mating wheel as above and observed as above, the only difference being that mating was not quantified for obvious reasons. The sample sizes used were: CS(23°)=15, CS(30°)=14, Ddc(23°)=27, Ddc(30°)=21. For some analyses, subsets of data were used with the sample size used indicated.

**Video Analysis**

Video analysis was done using a proprietary program developed especially for the purpose (source code and instructions available from N. Brandmeir). The following behaviors were monitored: male-following, wing extension, licking, attempted copulation, and copulation. For licking, attempted copulation, and wing extension a time
stamp was recorded measuring the time from introduction to the observed behavior. The number of stamps corresponded to the number of behaviors. For following and copulation a time stamp was recorded at the onset and termination of the behavior. Finally, if mating occurred and copulation terminated within 40 minutes, the males were monitored for courting again.

A time stamp in these experiments was the amount of milliseconds that had elapsed from the time of introduction until the observed behavior. The number of time stamps recorded was used to find the total number of behaviors observed in any particular category.

By analyzing the video in this way, it made it possible to monitor any changes that might occur in both rates of mating/courtship and also the rates of the individual steps of courtship described above. Also, because males will normally not court a mated female persistently due to her vigorous refusal signals, re-courtship was used as a metric for learning ability. The ability to learn to curb courtship displays based on female signals has been previously shown to be dependent on serotonin (Tempel and others 1984).

Statistics

Two-way ANOVAs were used to test for differences among groups with stock being one factor and temperature being the other. Any datum with a studentized residual $> |3|$ was considered an outlier and not included in the analysis except where noted. To test for a residual-treatment correlation a Brown-Forsythe test was used. To check for normality a Lillefors test was used. Any transformations necessary are indicated in the
body of the text and in the appropriate figure (Kutner and others 2005). Analyses comparing proportion data were done using $X^2$ analysis (Everitt, 1977).

**Figure 7: Schematic of Treatments Used in Mating Assays**

This schematic shows the treatments used in the mating assay. Only $Ddc$ flies would lack serotonin. Virgin CS females were used as mating ‘targets’ in all male-female experiments. CS males reared at 23°C were the ‘targets’ in all male-male trials.
Results

General Activity

This experiment was carried out in the manner described above. There was no significant difference observed between stocks or any difference in the mean boxes per minute due to the interaction between stock and temperature (i.e. the influence of serotonin loss). There was; however, a decrease in boxes per minute due solely to temperature. The average grid crossings or boxes entered (the number of times the fly crossed a gridline while being observed) decreased from 52.98 BPM to 42.15 BPM when the temperature was increased from 23°C to 30°C ($F=14.3, \text{df}=1,222, P=0.0002$) While the stock and the interaction between stock and temperature had no significant effect ($F_{\text{stock}}=3.36, \text{df}=1,222, P=0.07$ and $F_{\text{interaction}}=.84, \text{df}=1,222, P=0.36$) (Fig. 8).

Figure 8: Effects of Temperature and Genotype on General Activity – ($\bar{x} \pm 95\% \text{ CI}$) The only significant difference was due to temperature.
Courtship of Canton-S virgin females

This experiment measured the effect of temperature and genotype on courtship in Ddc\textsuperscript{1st} males. There were no significant differences observed between any of the treatment groups in the proportion of males courting ($X^2_{stock}=0.11$, df=1, $P=0.741$; $X^2_{temp}=2.37$, df=1, $P=0.124$; $X^2_{interaction}=3.00$, df=4, $P=0.558$), the proportion of males mating ($X^2_{stock}=3.19$, df=1, $P=0.074$; $X^2_{temp}=0.10$, df=1, $P=0.752$; $X^2_{interaction}=3.93$, df=4, $P=0.416$), or the proportion of courting males successfully copulating ($X^2_{stock}=3.41$, df=1, $P=0.065$; $X^2_{temp}=0.31$, df=1, $P=0.578$; $X^2_{interaction}=3.67$, df=4, $P=0.453$) (The sample sizes used for maters among courters were: CS(23°)=23, CS(30°)=27, Ddc(23°)=15, Ddc(30°)=21) (Fig 9, 10, & 11).

![Proportion of Flies Courting](image)

Figure 9: The Effects of Temperature and Genotype on the Proportion of Courtship

($\bar{X} \pm 95\%$ CI) There were no significant interactions found between any of the independent variables and the rate of courtship among the male flies.
Figure 10: The Effect of Temperature and Genotype on the Proportion of Mating – (x ± 95% CI) There were no significant interactions found between any of the factors and the rate at which the males successfully mated with virgin CS females.

Figure 11: The Effect of Temperature and Genotype on the Proportion of Mating among Courting Flies – (x ± 95% CI) There was no significant interaction observed. Neither temperature nor stock affected the rate of mating among males courting virgin CS females.
The interaction between genotype and temperature had a significant effect on wing extensions per minute (Fig 12) but not for any other index of courtship (Fig 13-16). Wild-type flies performed wing extensions at roughly the same rate at both temperatures while the Ddc flies decreased their average wing extensions per minute by 4.1 extensions per minute ($F_{stock} = 3.55$, $df=1.79$, $P=0.06$; $F_{temp} = 1.49$, $df=1.79$, $P=0.23$; $F_{interaction} = 9.97$, $df=1.79$, $P=0.002$) (The sample sizes used were: CS(23°)=23, CS(30°)=27, Ddc(23°)=15, Ddc(30°)=21) (Fig 12).

Temperature also had an effect on different aspects of courtship. Both the licks per minute and the duration of copulation were reduced by an increase in culture temperature (Fig 13 & 15). The average rate of licking at 23°C was 1.73 licks/minute while at 30°C the rate declined to 0.98 licks/minute ($F_{stock} = 0.10$, $df=1.77$, $P=0.75$; $F_{temp} = 10.16$, $df=1.77$, $P=0.002$; $F_{interaction} = 1.96$, $df=1.77$, $P=0.17$) (The sample sizes used were: CS(23°)=23, CS(30°)=27, Ddc(23°)=15, Ddc(30°)=21) (Fig 13). The duration of copulation also decreased, from a mean of 22.9 minutes at 23°C to 20.0 minutes at 30°C ($F_{stock} = 3.86$, $df=1.56$, $P=0.06$; $F_{temp} = 5.73$, $df=1.56$, $P=0.02$; $F_{interaction} = 0.21$, $df=1.56$, $P=0.65$) (The sample sizes used were: CS(23°)=11, CS(30°)=14, Ddc(23°)=11, Ddc(30°)=18) (Fig 15). No effects were noted for the rate of attempted copulations ($F_{stock} = 0.35$, $df=1.75$, $P=0.56$; $F_{temp} = 3.18$, $df=1.75$, $P=0.08$; $F_{interaction} = 3.79$, $df=1.75$, $P=0.06$) (The sample sizes used were: CS(23°)=23, CS(30°)=27, Ddc(23°)=15, Ddc(30°)=21) (Fig 14).
Figure 12: The Effect of Temperature and Genotype on the Rate of Wing Extension

(\bar{x} \pm 95\% CI) An interaction between $Ddc^{ol}$ and temperature caused the rate of wing extensions to be significantly lowered.

Figure 13: The Effect of Temperature and Genotype on the Rate of Licking

(\bar{x} \pm 95\% CI) Temperature caused a significant change in the rate of licks during courtship.
Figure 14: The Effect of Temperature and Genotype on the Rate of Attempted Copulations –
($\bar{x} \pm 95\%$ CI) There were no significant interactions between any factor and the number of attempted copulations per minute of following.

Figure 15: The Effect of Temperature and Genotype on the Duration of Copulation –
($\bar{x} \pm 95\%$ CI) Temperature caused a significant effect on the length of copulations.
Figure 16: The Effect of Temperature and Genotype on Time until Mating –

(\bar{x} \pm 95\% CI) Temperature had no significant effect on the time it took for flies to mate once introduced.

Only one difference was observed between CS and Ddc flies independent of temperature. That difference was in the time it took for the flies to mate after being introduced (Figure 16). The wild-type flies took an average of 12.27 minutes to mate after being introduced to a female. The Ddc flies, on the other hand, took an average of 7.12 minutes (F_{\text{stock}}=9.40, df=1,56, P=0.003; F_{\text{temp}}=0.17, df=1,56, P=0.68; F_{\text{interaction}}=0.08, df=1,56, P=0.78) (The sample sizes used were: CS(23°)=15, CS(30°)=17, Ddc(23°)=12, Ddc(30°)=18).

**Homosexual Courtship**

There was no significant interaction between the rearing temperature and the strain, though culture temperature did produce a significant effect. Flies cultured at 23°C were never observed courting CS males. Flies kept at 30°C courted CS males in 17.14%
of the interactions ($X^2_{\text{stock}}=0.012, \text{df}=1, P=0.91; X^2_{\text{temp}}=6.42, \text{df}=1, P=0.011$; $X^2_{\text{interaction}}=9.39, \text{df}=4, P=0.0521$) (Fig 17) (For a summary of all results see Table 3).

**Figure 17: The Effect of Temperature and Genotype on the Percentage of Homosexual Courtships** – ($\bar{x} \pm 95\% \text{ CI}$) Only temperature had a significant effect on the percentage of flies participating in homosexual courtship.
<table>
<thead>
<tr>
<th>Behavior</th>
<th>CS(23°) Mean ± 95%CI</th>
<th>CS(30°) Mean ± 95%CI</th>
<th>Ddc(23°) Mean ± 95%CI</th>
<th>Ddc(30°) Mean ± 95%CI</th>
<th>P_{stock}</th>
<th>P_{temp}</th>
<th>P_{interact}</th>
</tr>
</thead>
<tbody>
<tr>
<td>General activity</td>
<td>60.6 ± 6.64</td>
<td>44.5 ± 7.38</td>
<td>51.2 ± 5.28</td>
<td>41.3 ± 4.08</td>
<td>0.07*</td>
<td>0.0002*</td>
<td>0.36*</td>
</tr>
<tr>
<td>% Courting</td>
<td>92.3 ± 10.7</td>
<td>87.1 ± 14.4</td>
<td>88.9 ± 15.24</td>
<td>84.0 ± 14.96</td>
<td>0.12**</td>
<td>0.74**</td>
<td>0.56**</td>
</tr>
<tr>
<td>% Mating</td>
<td>57.7 ± 19.8</td>
<td>48.6 ± 17.14</td>
<td>66.7 ± 22.8</td>
<td>72.0 ± 18.3</td>
<td>0.75**</td>
<td>0.75**</td>
<td>0.416**</td>
</tr>
<tr>
<td>% Courters mating</td>
<td>62.5 ± 20.2</td>
<td>62.9 ± 18.9</td>
<td>75 ± 22.2</td>
<td>85.7 ± 15.64</td>
<td>0.07**</td>
<td>0.58**</td>
<td>0.45**</td>
</tr>
<tr>
<td>Wing ext. per min.</td>
<td>5.93 ± 2.04</td>
<td>7.75 ± 1.62</td>
<td>10.7 ± 2.58</td>
<td>6.56 ± 1.30</td>
<td>0.06*</td>
<td>0.23*</td>
<td>0.002*</td>
</tr>
<tr>
<td>Attempted copulations per min.</td>
<td>1.57 ± 0.56</td>
<td>0.66 ± 0.24</td>
<td>1.24 ± 0.52</td>
<td>1.28 ± 0.56</td>
<td>0.56*</td>
<td>0.08*</td>
<td>0.06*</td>
</tr>
<tr>
<td>Licks per min.</td>
<td>1.56 ± 0.44</td>
<td>1.11 ± 0.46</td>
<td>1.99 ± 0.78</td>
<td>0.83 ± 0.38</td>
<td>0.75*</td>
<td>0.002*</td>
<td>0.17*</td>
</tr>
<tr>
<td>Minutes until mating</td>
<td>12.1 ± 4.50</td>
<td>12.4 ± 3.82</td>
<td>6.38 ± 2.20</td>
<td>7.57 ± 2.18</td>
<td>0.003*</td>
<td>0.68*</td>
<td>0.78*</td>
</tr>
<tr>
<td>Copulation duration</td>
<td>21.4 ± 1.69</td>
<td>18.9 ± 2.72</td>
<td>24.5 ± 3.46</td>
<td>20.8 ± 2.08</td>
<td>0.06*</td>
<td>0.02*</td>
<td>0.65*</td>
</tr>
<tr>
<td>% Courting homosexually</td>
<td>0 ± 0.20</td>
<td>14.3 ± 1.08</td>
<td>0 ± 0.18</td>
<td>19.0 ± 1.08</td>
<td>0.91**</td>
<td>0.011**</td>
<td>0.052**</td>
</tr>
</tbody>
</table>

Table 3: Summary of Results - * Values obtained with 2-way ANOVA. **Values obtained with $X^2$ tests.
Discussion

Temperature

Surprisingly, these experiments showed that the temperature that *Drosophila* males are cultured at has many effects on their general activity and their courtship behavior. Culturing at 30°C reduced the rate of licking, general activity, and the length of copulation. Increased temperature also caused homosexual courtship toward mature males (no homosexual courtship was recorded amongst flies reared at 23°C). Temperature change can have broad effects on exothermic organisms. The origins for these behavioral changes could be metabolic or brought about by differing environmental cues rising from a warmer environment. For example, a warmer environment may cause an abundance of olfactory stimuli in the culture to increased evaporation of substrate and communicative compounds. This, in turn, could cause a decrease in gustatory behavior eventually leading to a decrease in licking behavior during courtship.

In any event, this result demands further investigation. Temperature sensitive mutations are a common and invaluable tool in *Drosophila* and effects caused simply by temperature alone have a huge potential to confound various results. At the very least, it underscores the absolute necessity of using wild-type flies reared at permissive and restrictive temperatures as controls in all experiments relying on temperature sensitive mutations.

Strain

*Ddcยก* flies mated with a lower latency than CS flies, regardless of temperature. This trait is known to vary among strains and the fact that the result was constant across temperature change indicates that the Ddcยก allele was likely not the cause. More likely,
the genetic background of each strain contained sufficient differences to cause the effect observed.

**Serotonin**

$Ddc^{st1}$ flies and only $Ddc^{st1}$ flies lack physiologically significant levels of serotonin when cultured above 29°C. Thus, any effect caused by having the $Ddc^{st1}$ allele and being cultured at 30°C can be inferred as an effect caused by a lack of serotonin.

The experiments presented above go a long way in describing the role serotonin plays in the courtship behavior of *Drosophila* males. Since there was no affect of culturing at restrictive temperature and expressing the $Ddc^{st1}$ allele on rate of courtship, rate of mating, rate of mating among courters, time from introduction until mating, duration of copulation, rate of licking, rate of attempted copulations, or the rate of homosexual courtship it must be concluded that serotonergic neurons do not play an essential role in generating those behaviors. These results confirm some earlier observations (Tempel and others 1984) that also showed no decrement in mating among heat treated $Ddc^{st1}$ flies while conflicting with anecdotal results presented by others (Yamamoto, 1997).

The experiments also showed that serotonin was integral in the rate of wing-extensions or songs performed by the male during courtship. This result hints at a system regulating song analogous to that which regulates heartbeat. It is known that *Drosophila* heartbeat is controlled by ion channel oscillators (Dowse and others 1995) that are in turn modulated by neurotransmitters including serotonin (Johnson and others 2002). Ion channel oscillators found in the heart are also involved in generating the courtship song of *Drosophila* (Hall 1994). Perhaps then, similar mechanisms modulate heartbeat and
courtship song. Hall hypothesized that the courtship song provides stimulation not only to the female, but also to the male generating the song through a positive feedback system (JC Hall, pers. comm.). If this is the case, the lack of serotonin could have perturbed song production, causing subnormal levels of positive feedback to the male singer. Since this did not decrease the males' average success in mating, it is seems that some parts of the courtship song may be for male stimulation and others for female stimulation. Also, it is likely that other neurotransmitters play a role in song production too. Studies testing the role of various neurotransmitters on courtship song and its effect on both the singer and female are certainly warranted.
REFERENCES


BIOGRAPHY OF THE AUTHOR

Nicholas Brandmeir was born on July 15, 1980 in Bangor, ME. He graduated from Palisade High School in Palisade, CO in 1998. He earned a Bachelor of Arts in Biological Sciences from Cornell University in Ithaca, NY in May, 2002. He was married to Cheryl Holleran of Severna Park, MD in June, 2005. After receiving his degree, Nicholas will be attending Albany Medical College to begin training as a physician. Nicholas is a candidate for the Master of Science in Zoology degree from the University of Maine in August, 2006.