

**OPTIMALITY AND COURTSHIP
BEHAVIOR IN ZEBRAFISH, *DANIO RERIO***



5TH YEAR INTEGRATED M.S DISSERTATION

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DECLARATION

We hereby declare that the work reported in this thesis is entirely original and was carried out in the zebrafish behavior laboratory in the Department of Biological Sciences of the Indian Institute of Science Education and Research – Kolkata. We further declare that it has not formed the basis for the award of any degree, diploma or similar title of any University or other institution.

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*To mate or not to
mate: THAT is the
question:*

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1. Abstract

Behaviors involving repeated investments on the part of the actor and diminishing marginal returns from these investments are seen to be optimized by maximizing the rate of returns from the behavior. This idea, known as the marginal value theorem, was initially used to study foraging strategies in animals when food is available in patches in an environment. In this study, we adopt the marginal value theorem and utilize it to study courtship behavior in zebrafish *Danio rerio* (with males being equivalent to foraging animals and females being compared to patches of resources). Our application of the marginal value theorem to courtship in zebrafish predicted that a male spends less time courting a female if the female density in the environment is high and *vice-versa*. The results of our experiments support these predictions and show that courtship time in zebrafish is indeed greatly influenced by the female density in the environment. Additionally, we went on to construct a return function in the form of the probability that a female being courted mates with the male courting her as a function of the time invested by the male in courtship (equivalently, the number of times the male physically interacts with the female). This return function, in a sense, gives an idea of the sexual response of a female as the investment in courtship from the side of a male increases and can potentially prove the existence of diminishing marginal returns from repeated investments in courtship on the part of the male.

2. Introduction

The quest for optimality critically shapes animal behavior. With a Darwinian view of natural selection in mind (Darwin, 1859), every behavior is inevitably linked to its adaptive value. The aim, therefore, running through behavioral biology is to establish the adaptive significance of animal behavior in addition to the proximate understanding of how a particular behavior affects the actor and the recipient (Tinbergen, 1963). The ultimate adaptive question about animal behavior thus deals with the evolutionary process which shapes the behavior so that it maximizes the benefit/return to the actor.

It is easy to see how a behavior may be adaptive if it involves a single, one-time investment on the part of the actor. Darwin himself (Darwin, 1859), and other adaptive evolutionists (Dawkins, 2009) have understood that such a behavior, if adaptive, would tend to maximize the return (and thereby, the reproductive fitness) to the acting organism. Behaviors which involve multiple repeated investments are however not subject to such a paradigm. It was explicitly shown by Eric Charnov (Charnov, 1976), and demonstrated in laboratory and field studies on foraging (Krebs et al. 1974; Krebs et al., 1977) that such behaviors would look to maximize the rate of returns to the actor. This theorem is known as the Marginal Value Theorem in behavioral biology, and has been utilized to understand optimal foraging in a variety of animals (Krebs et al., 1974; Krebs et al., 1977), and even nutrient seeking growth of plant roots (McNickel and Cahill, 2009). Humans attempting to remember and search for memories have also been recently studied under the framework of optimal foraging and the marginal value theorem (Hills et al., 2012).

Male courtship behaviors, across sexual organisms, typically involve repeated investments (in the form of time spent courting the female) on the part of the male. The return, in this case, can be visualized as the probability that the female decides to mate with the male. With this understanding, it is expected that courtship behaviors will also be subject to the marginal value theorem, and follow its predictions. However, most studies of optimal mating (few in number) have involved the observation of the mating act itself under the purview of the marginal value theorem. They have attempted to look at the optimal mating latency (duration of the mating bout) and its dependence on the density of females in the observed group of organisms (Parker et

al., 1993; personal observations with *Drosophila melanogaster*). Studies attempting to understand courtship, in the light of optimality under the marginal value theorem have been very few and far between.

The present study aims to look at the duration of male courtship behavior and its dependence on the density of females in zebrafish *Danio rerio* in the light of the marginal value theorem. Zebrafish, being an externally fertilizing species, offer a unique opportunity to dissect the applicability of the marginal value theorem in male courtship behavior. Since fertilization occurs externally in zebrafish, it is reasonable to assume that mating bouts with individual females are approximately of the same duration. It is then possible to clearly establish the effect of female density on the courtship duration, and its prediction under the purview of the marginal value theorem.

Courtship behavior in male zebrafish has been characterized in quite a few studies (Darrow et al., 2004; Spence et al., 2006a; Spence et al., 2008). It typically involves the male initially chasing the female and performing certain wiggling motions. The male also nudges the female, which would result in the female laying eggs if she is interested in spawning. An uninterested female will move away from the nudging male – this may lead to the male re-instating his efforts to attract the female by chasing her. At each instant of time during the courtship bout, the male needs to decide if he should continue to invest more time in courting the same female – the male can potentially leave the current female he is courting and go out in search of a new mating partner. It is this process of continuous decision making, involving repeated investments of time, which makes the study of male courtship amenable under the framework of the marginal value theorem.

The marginal value theorem, to be applicable, assumes diminishing returns from repeated investments (Charnov, 1976). In the case of male courtship behavior, this can be interpreted as an assumption that the differential increase in the probability that the female will decide to spawn becomes smaller as the time invested by the male in courting the female increases. This assumption can obviously be validated if the predictions of the marginal value theorem regarding the effect of female density on time invested by a male in courtship are upheld in experimental observations. In this study, however, we go on to additionally note down the occurrence of

mating/spawning events and attempt to plot the returns from courtship as a function of time investment in an effort to see if they are indeed diminishing in nature.

3. Effect of female density on male courtship duration

3.1 Marginal value theorem as applied to courtship in zebrafish

For a behavior to be studied under the purview of the marginal value theorem, the following two conditions need to be satisfied (Charnov, 1976):

1. Repeated, multiple investments should be involved on the part of the actor.
2. There should be diminishing returns from the investments.

As in behaviors involving a single investment-return relation, the optimal behavior in a repeated investment case eventually aims to maximize the total return from the behavior. Taking dR to be the differential return from a differential investment of time dt , the total return from the behavior can be written as the integral of the differential returns over the time interval t during which the behavior is performed (as in Charnov, 1976):

$$\text{Total return } R = \int_0^t dR = \int_0^t \frac{dR}{dt} dt \quad (3.1)$$

It is clear from this integral form of the return function that the total return R from a behavior which involves repeated investments of time will be greatest if the rate of returns $\frac{dR}{dt}$ is maximized. This conclusion, in combination with the assumptions of repeated investments of time and diminishing marginal returns from these investments, constitutes the framework of applicability of the marginal value theorem. To study courtship behavior in zebrafish in the light of optimality, we therefore need to first confirm if the behavior conforms to the requirements of the marginal value theorem.

It is obvious that courtship involves a repeated time investment on the part of the male – at each instant of time, the male needs to decide afresh if he should invest more time courting and chasing the same female. The fact that the returns (in the form of the probability that the female will mate with the courting male) diminish with the investment of time indeed needs to be established explicitly. However, we do realize that this mating probability initially increases quickly as the male invests more time in courtship, but the rate of increase decays as time goes

on (the female could be disinterested in mating with the courting male). Eventually, the mating probability has to asymptotically approach 1 (it cannot exceed 1) – this inference itself demonstrates that the marginal increase in the mating probability has to decrease as the time invested by the male in courtship increases.

Taking a representative explicit example to model this situation, we consider the rate of change of the probability that a female will mate with a courting male to be proportional to the difference between the current value of the mating probability and 1 (the maximum value of the mating probability). In this case, as required, rate of increase of the mating probability decays as time goes on and the probability approaches 1. Thus if P is the mating probability and c is the constant of proportionality, we can write:

$$\frac{dP}{dt} = c(1 - P) \tag{3.2}$$

Separating variables and integrating both sides of the equation, we get:

$$P = 1 - e^{-ct} \tag{3.3}$$

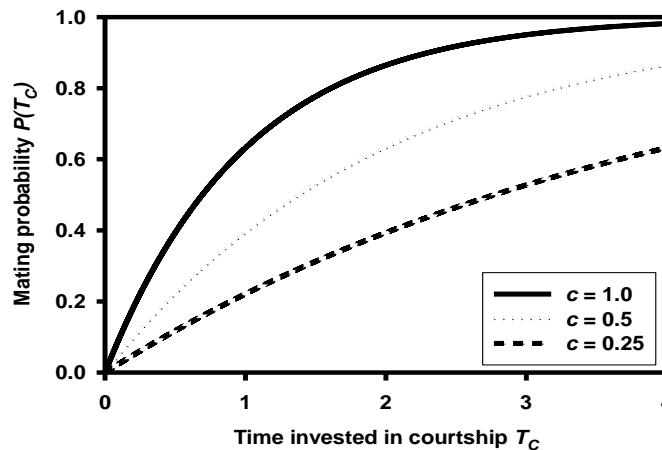


Figure 3.1

From Figure 3.1, it can be seen in this case, the differential increase in the mating probability diminishes as the time invested by the male in courtship increases and the probability approaches 1 asymptotically. The constant c can be understood as the rate at which the mating probability

increases initially (when $P \approx 0$) – if c is small, the initial increase in the probability function is slow. However, irrespective of the value of c , the function approaches 1 asymptotically.

Any mating probability function of this form allows us to apply the marginal value theorem to study male courtship behavior – although the exact nature of the mating probability may be somewhat different, we will use the function discussed in Figure 3.1 taking $c = 1$ to graphically highlight the predictions which arise out of the marginal value theorem. In our analytical calculations, however, we will use a generalized form of the mating probability function $P(t)$ to make it explicitly clear that such an analysis is perfectly applicable to all forms of the mating probability as long as it involves diminishing marginal/differential returns from repeated investments of time.

Starting the analysis on the marginal value theorem framework, we let:

Time spent searching for the female = T_S

Time spent mating with the female = T_M

Time spent courting the female = T_C

Time spent actually mating = T_{AM}

$$T_M = T_C + T_{AM}$$

Return function = $F(T_M)$

Probability function = $P(T_C)$

$$F(T_M) = \frac{P(T_C)}{T_S + T_M} = \frac{P(T_C)}{T_S + T_C + T_{AM}} \quad (3.4)$$

Since fertilization is external in zebrafish, we can reasonably assume that T_{AM} is the same in all cases and therefore, account for it within T_C . T_C is then the main contribution to the male investment. In this scenario, the return function becomes dependent on T_C instead of T_M as follows:

$$F(T_M) = \frac{P(T_C)}{T_S + T_C} \quad (3.5)$$

The marginal value theorem now requires the maximization of the return function $F(T_M)$ as in (3.5):

$$F'(T_C^*) = \frac{P'(T_C^*)(T_S + T_C^*) - P(T_C^*)}{(T_S + T_C^*)^2} = 0$$

This yields the critical courtship time T_C^* as:

$$T_C^* = \frac{P(T_C^*)}{P'(T_C^*)} - T_S \quad (3.6)$$

We now claim that this critical time T_C^* , which maximizes the rate of returns to the male, can be read off from the graph of the mating probability function versus the time invested by the male in courtship shown in Figure 3.1 with $c = 1$. This critical time is shown in a modified form of Figure 3.1, as below:

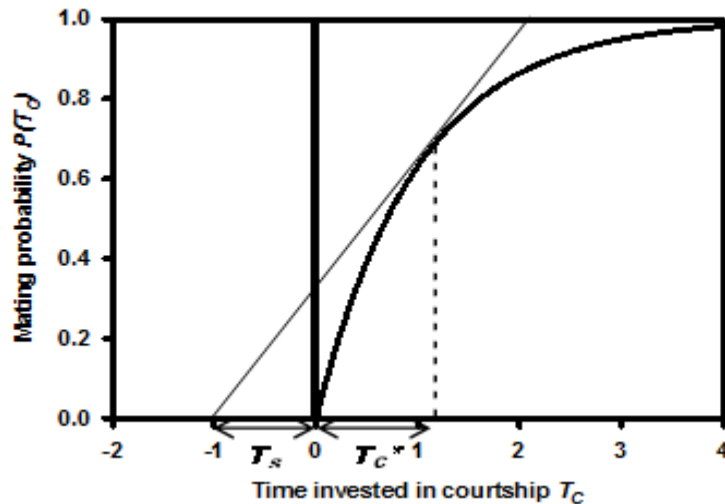


Figure 3.2

The critical time T_C^* and the value of the probability function $P(T_C^*)$ at that time is indicated in Figure 3.2. T_C^* is given by the point of intersection between the straight line tangent to $P(T_C)$ and passing through the points $(-T_S, 0)$ and $(T_C^*, P(T_C^*))$ as shown in Figure 3.2. To understand this claim, let us attempt to find the slope of this straight line. The slope will be:

$$m = \frac{\Delta y}{\Delta x} = \frac{P(T_C^*)}{T_S + T_C^*}$$

Since this straight line is tangent to $P(T_C)$ at T_C^* , its slope is also equal to $P'(T_C^*)$. Equating this to m , we get:

$$m = \frac{P(T_C^*)}{T_S + T_C^*} = P'(T_C^*)$$

This yields,

$$T_C^* = \frac{P(T_C^*)}{P'(T_C^*)} - T_S \quad (3.7)$$

We see that this expression for the critical time is identical to that derived previously in (3.6) showing that the critical courtship time can indeed be read off from the mating probability curve, if the search time T_S is known.

Having worked out this graphical way of interpreting the critical time for courtship, we go on to see its variation with changes in the time spent in searching for a female T_S , as follows:

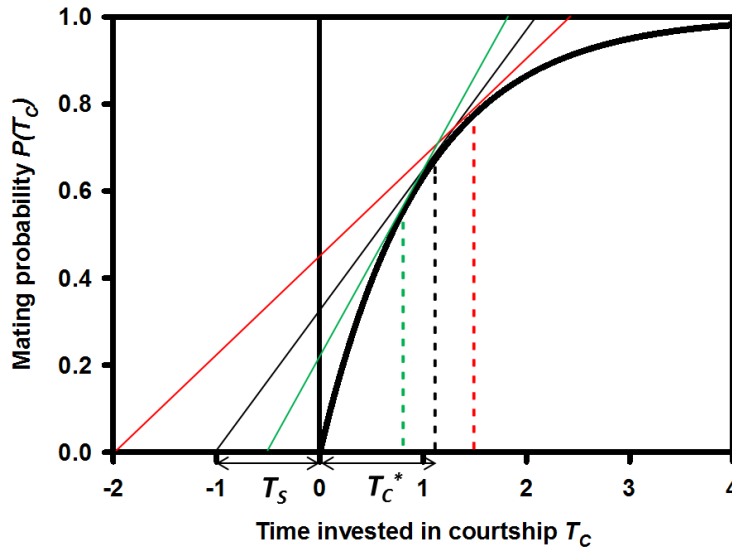


Figure 3.3

Figure 3.3 shows two cases – one with a longer search time (in red) and the other with a shorter search time (in green). We can clearly see from the figure that the critical courtship time is

shorter than previously when the search time is shorter (green) and longer when the search time is longer (red). The previous search time, and critical courtship duration are marked as T_S and T_C^* respectively.

Since the time spent by the male in searching for a female would be longer if the number of females present is smaller and vice versa (assuming all other environmental factors to be unchanged), the marginal value theorem yields testable predictions about the male courtship duration as a function of the density of females present. It predicts that this duration would be smaller when the density of females is high and higher when the density of females is low. This prediction emanating from the marginal value theorem was subjected to experimental scrutiny as part of this study which we now describe.

3.2 Materials and methods

3.2.1 Zebrafish populations:

The population of zebrafish *Danio rerio* used in our experiments was procured from a local fish vendor in Kanchrapara in North 24 Parganas district (West Bengal, India). This population of adult fishes had been maintained by the aforementioned vendor in his aquarium facility for a period of two-three months before our purchase. This is a mixed population of zebrafish isolated from nearby stagnant water bodies located in Halisahar (District North 24 Parganas, West Bengal, India) and Ranaghat (District Nadia, West Bengal, India).

3.2.2 The observation room and experimental arena

For setting up the experimental arena, a fish tank of dimensions 48"×12"×9" was divided into several sections as shown in Figure 3.4 below:

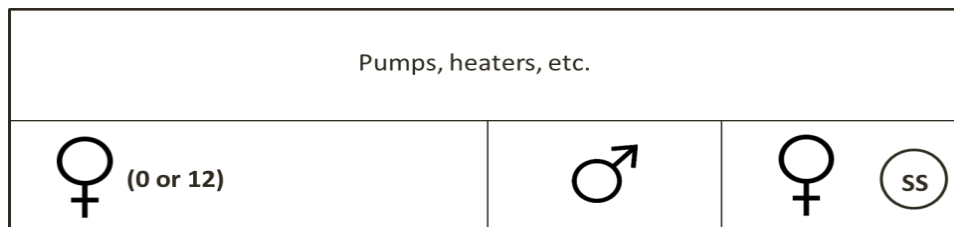


Figure 3.4

As shown in Figure 3.4, a partition was inserted to divide the tank into two equal halves along its length. One of the resultant chambers was utilized for setting up aquaria instruments such as air pumps and heaters to maintain air circulation within the aquarium water and regulate its temperature. A thermometer to measure the water temperature was also set up in this chamber. The other long chamber was further subdivided into two equal sized halves – one of these sub-chambers housed a variable number of female zebrafish (0 or 12 depending upon the experimental set). Its equivalent counterpart chamber was further subdivided into two more smaller equal sized chambers – the chamber closest to the one housing a variable number of female zebrafish was utilized to house a lone male zebrafish. The chamber farther away housed a lone female zebrafish and a removable spawning site (SS in Figure 3.4). The spawning site consisted of a petri plate filled with moderate sized gravel. Female zebrafish have been previously seen to preferentially oviposit in places which can potentially provide shelter and protection to the fertilized eggs after they are laid – the presence of gravel (the eggs, as they are laid, get hidden and sheltered between the pieces of gravel) in the spawning site has been shown to result in significant preference during oviposition (Lawrence, 2007; Spence et al., 2007). Consequently, it is expected that most of the spawning would occur over this petri plate. All the partitions used to create the various chambers were transparent and removable – this resulted in open exchange of water between the different chambers of the tank. Such exchange of aquarium water and unhampered vision through transparent partitions ensured free communication through visual, olfactory and hormonal cues which are known to play an important role in zebrafish spawning (Chen and Nartinich, 1975; van den Hurk and Lambert, 1983; Gerlach, 2006). However, the fish were unable to move out of the chamber in which they were housed unless a partition was removed.

The temperature in the observation room, which housed the experimental setup, was maintained in a range of 24–30 °C which is considered as the optimum for rearing zebrafish (Matthews et al., 2002). Additionally, a 14:10 hr light-dark (LD) cycle was maintained in the room with lights coming on at 0800 hr and going off at 2200 hr each day. A long photoperiod LD cycle has been previously seen to promote spawning – zebrafish oviposition circadian rhythms are seen to entrain to a 14:10 hr LD cycle (Blanco-Vives et al., 2009).

3.2.3 Setting up of experiments and video recordings

Before setting up spawning experiments with different densities of females in the arena to test the predictions of the marginal value theorem, we separated out males and females from the original stock populations and kept them isolated as two different populations in the observation room for 7-10 days prior to the start of experiments. Sexual separation in this form is often seen as a positive factor to encourage spawning in zebrafish when the sexes are brought together – a long separation period of the order of 15-20 days was, however, avoided as it can result in females getting egg-bound and unable to oviposit thereafter (Spence et al., 2008). The tanks housing these separated populations were covered on three sides with brown paper in order to prevent the exchange of visual communication cues.

Zebrafish are known to spawn primarily within the first hour of light each day both in the laboratory (Selman et al., 1993) and in the wild (Spence et al., 2006b). Consequently, we removed the partition between the lone male and female in the arena as soon as lights came on at 0800 hr each day and recorded the behavior of the fishes for the next 30 minutes. The experimental arena was set up in the evening prior to the recording in order to allow the fishes to get accustomed to their surroundings overnight. The experiment was repeated multiple times in two different sets – one set had no females and the other had 12 females in the large chamber housing a variable number of females as shown in Figure 3.4. Additionally, there were two separate identical arenas in which experiments were simultaneously performed each day.

Since the fishes are expected to primarily spawn over the spawning site (SS in Figure 3.4) containing gravel (Lawrence, 2007; Spence et al., 2007), we went on to manually analyze the recorded videos and noted down the total time which the male and female spent together in close proximity to the spawning site (when the male, female and spawning site would be within one body length of each other). This time, as a fraction of the total recording time each day, gave us a measure of the time spent by the male in courting and mating with the lone female in two different situations – one with a single female and the other with 13 females in total in the arena. We then compared this time, under the two different female densities, in order to test compliance with the predictions of the marginal value theorem. At the conclusion of each trial, we additionally checked the spawning site for the presence of fertilized eggs – recordings from the days when eggs were not seen in the spawning site were left out from the final analysis.

A single set of the male and females was not used in more than two consecutive experimental days. The males and females used in an experiment were separated once again after the conclusion of the experimental run and were kept isolated for 7-10 days before being allowed to participate in experiments again.

3.2.4 Statistical analyses

The percentage time spent by the male courting and mating with the female and the number of eggs laid in the spawning site under the two different female densities were statistically compared using the Mann-Whitney U test. $p < 0.05$ was considered as the level of statistical significance. The statistical analyses were implemented using the statistical module of SigmaPlot (version 11.0) for Windows (Systat Software Inc., 2008).

3.3 Results

The percentage time spent by the male around the female physically accessible to it in close proximity to the spawning site given two different total number of females (1 or 13) in the experimental tank is shown in Figure 3.5 below:

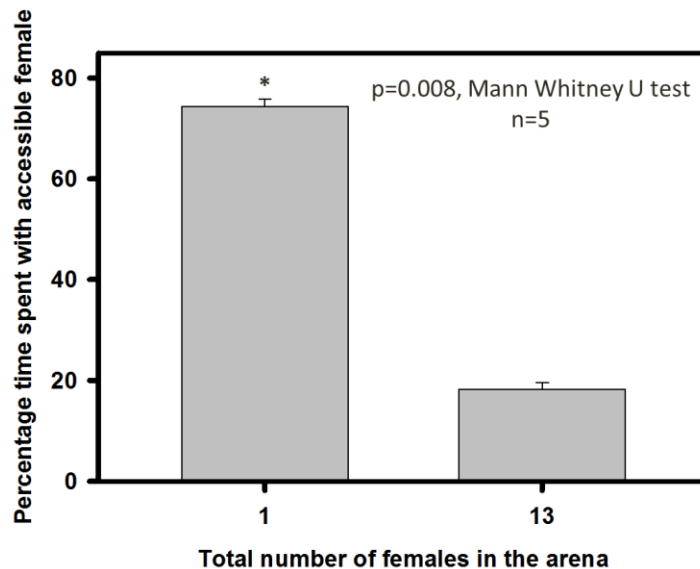


Figure 3.5

The percentage time of the total recording time each day spent by the male in proximity with the accessible female and the spawning site is seen to be $74.4 \pm 1.4\%$ (mean \pm SEM) when there is

only one female in the experimental tank. This percentage decreases to $18.22 \pm 1.32\%$ (mean \pm SEM) when the experimental arena houses 13 females in total. The Mann-Whitney U test reveals a significant difference between the percentage time under the two different female densities ($p=0.008$). This difference is in accordance to the marginal value theorem which had predicted that the percentage time would be smaller if there were a larger number of females in the environment (and hence, the time that the male would need to invest in searching for a female would be small) and *vice-versa*.

However, we do not see any significant difference between the number of eggs laid in the spawning site under the two female densities ($p=0.421$). 74.6 ± 16.9 and 47 ± 10.7 eggs (mean \pm SEM) are seen to be laid when there are a total of 1 and 13 female in the experimental tank respectively (shown in Figure 3.6 below):

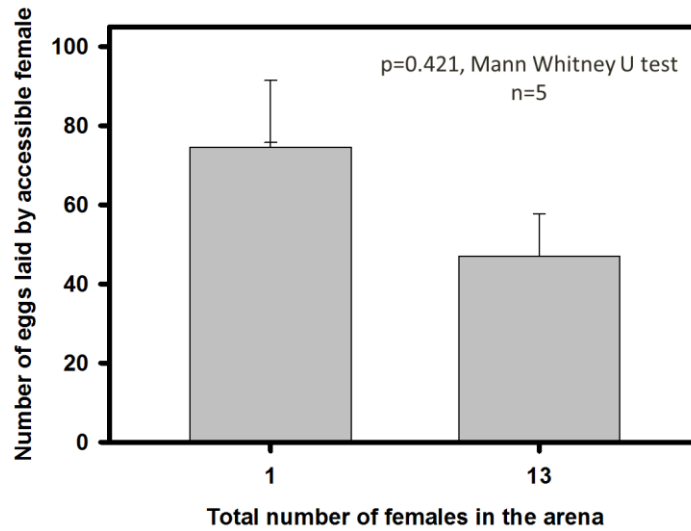


Figure 3.6

Figure 3.7 shows a scatter plot of the number of eggs laid by the female physically accessible to the male against the percentage time spent by the male in proximity to this female and the spawning site. As expected from the conclusion in Figures 2.5 and 2.6, we can clearly distinguish a big difference between the two female densities along the percentage time axis but no such separation along the number of eggs axis. However, the large variance in the number of eggs laid in both the experimental setups shows that it is important to get data from a larger number of replicate experiments to be able to reach a clear conclusion on this issue.

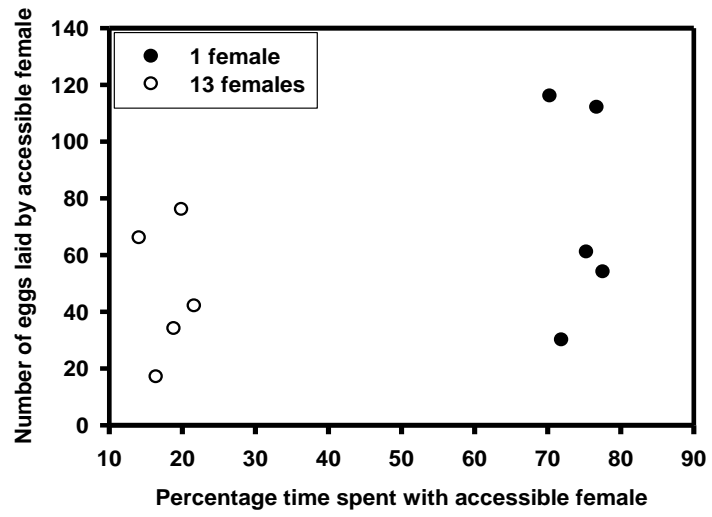


Figure 3.7

4. Investment-return relation in courtship behavior

The marginal value theorem, to be applicable in the case of a behavior involving repeated investments on the part of the actor, assumes that the marginal/differential returns from these investments diminish. In the previous chapter, we showed that the differential returns (in the form of the probability that a female decides to mate) from repeated investments of time on the part of a male in courting a female are most likely diminishing in nature. With this understanding in mind, we went ahead to apply the marginal value theorem to the study of courtship behavior in zebrafish and came up with experimentally testable predictions regarding the time that a male would invest in courting a female as a function of the total number of females in the environment. The results from these experiments were seen to uphold the predictions from the framework of the marginal value theorem, and thereby, yield clear support to the idea of diminishing marginal returns from repeated time investments during male courtship behavior.

It would, however, be very interesting to attempt to experimentally find out the marginal returns (mating probabilities) as a function of the time invested by a male in courting a female and visualize the exact nature of this return function. In this part of the study, therefore, we aim to carry out suitable observations designed to eventually yield the return function in the form of mating probabilities and check if the marginal returns indeed diminish as the time invested in courtship keeps on increasing.

4.1 The quantity to be experimentally measured and the form of the dataset

Ideally, we would like to plot the probability that the female being courted decides to mate with the courting male as a function of the time that the male invests in courtship. However, in actual observations, we find that individual interactions between the male and female are very short in nature and it is very difficult to note down the lengths of these individual interactions without significant variation across observers. During the courtship ritual, however, the male physically pushes and nudges the female with his snout in an attempt to urge her to spawn (Darrow et al.,

2004). We find that it is possible to uniquely count the number of these physical interactions and use it as a surrogate for the time invested by the male in courtship. This use of a surrogate is reasonable because it is clear that as the time invested by the male in courtship increases, the number of these physical nudges and pushes increase too – therefore, a direct proportionality between these two quantities exists. Consequently, we went on to collect data so as to be able to plot the returns in the form of mating probabilities as a function of the number of physical interactions between the male and the female instead of the time invested by the male in courtship.

With the above ideas in mind, we recorded the behavior of the male and female at a resolution high enough to enable us to clearly see and uniquely note down the occurrence of a spawning event. We followed this by counting the number of male-female physical interactions between two consecutive spawning events. Thus, our data set finally consisted of a string of physical interactions in time interspersed with spawning instances, as shown in Figure 4.1 below with dashes indicating physical interactions and S indicating spawning events:

S-----S-----S--S-----S-----S--S-----S-----S

Figure 4.1

4.2 Materials and methods

The zebrafish populations used and conditions maintained in the observation room are identical to those described in the previous chapter.

4.2.1 The experimental arena

We housed the fishes in a tank of dimensions 7.5"×4"×8" shown as follows in Figure 4.2:

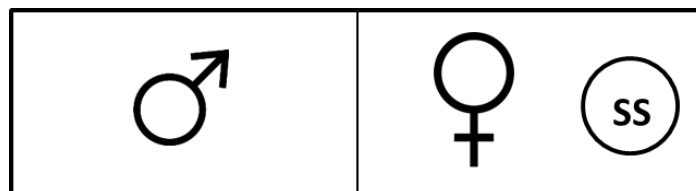


Figure 4.2

A transparent partition separated the experimental tank into two equally sized chambers along its longer dimension housing a male and a female respectively. The spawning site was placed on the side of the female as described in the previous chapter.

4.2.2 Setting up of experiments and video recordings

The experimental protocol and the procedure of recording the behavior of the male and the female was very similar to that described in the previous chapter. In this part of the study, however, we recorded behavior at a much higher resolution than previously in order to enable us to identify oviposition events clearly. Additionally, it was possible to cover the entire experimental arena in these recordings – consequently, we could note down any rare spawning events which happened outside the spawning site as well.

The recorded videos were then manually analyzed by two independent observers who noted down the times at which spawning took place as well as the number of physical interactions between consecutive spawning events. This generated the data set in the form we described in Figure 4.1. Any discrepancy between the counts of physical interactions noted down by the two independent observers was resolved by taking the average of the two observations and rounding it off to the closest whole number.

4.2.3 Analysis of the data set and plotting the return function

After generating the data set in the form shown in Figure 4.1 from our experiments, we went on to plot the frequency distribution $f(n)$ of the number of male-female physical interactions interspersed between two consecutive spawning instances n . The normalized form of this frequency distribution therefore gave us the probability distribution $p(n)$ of the inter-spawning number of physical interactions between the male and the female n .

However, $p(n)$ is clearly not the return function (as probability of mating/spawning) we are attempting to plot in this part of the study. The return function (with the return being the probability that a female will mate with the male courting her) we are trying to find actually plots the probability of the number of physical interactions n between consecutive mating events *given that* the previous $n-1$ interactions have been unsuccessful (they have not resulted in spawning). Therefore, the return function is actually a conditional probability distribution which we have to derive from the distribution $p(n)$ which we now proceed to do.

Using the definition of conditional probability, we can write:

$$p(n|n - 1 \text{ unsuccessful}) = \frac{p(n, n - 1 \text{ unsuccessful})}{p(n - 1 \text{ unsuccessful})} \quad (4.1)$$

Since the events where two consecutive spawning instances were separated by n physical interactions are also the ones where the previous $n-1$ have been simultaneously unsuccessful, we can now write:

$$p(n, n - 1 \text{ unsuccessful}) = p(n) = \frac{f(n)}{\text{Total frequency}} \quad (4.2)$$

Also, all the instances where the number of physical interactions between two consecutive mating events is *greater than or equal to* n are those where $n-1$ interactions have been unsuccessful in causing the fishes to spawn. Therefore:

$$p(n - 1 \text{ unsuccessful}) = p(N \geq n) = \frac{f(N \geq n)}{\text{Total frequency}} \quad (4.3)$$

Putting (4.2) and (4.3) back into (4.1), we get:

$$p(n|n - 1 \text{ unsuccessful}) = \frac{f(n)}{f(N \geq n)} = \text{Return function } r(n) \quad (4.4)$$

The return function $r(n)$ which we want to plot is therefore of the form given in (4.4).

4.3 A representative simulation

We started off our study of the return function by carrying out a representative computer simulation in order to create a baseline against which our experimental observations could be compared. In this simulated experiment, we assumed fishes to be moving randomly inside the experimental arena and considered their collisions to be equivalent to physical interactions like nudges and pushes. We assumed that the underlying distribution of the probability that a female being courted will mate with the courting male as a function of the number of physical interactions between them is diminishing in nature. By comparing the return function obtained by analyzing the data generated by the simulation to the mating probability distribution fed in *a priori*, we could additionally confirm that the calculations we are employing to generate the

return function from the initial frequency distribution (as shown in the previous section) are correct. Greater details regarding the parameters used in this simulation can be obtained from the copy of the code of the program attached in appendix A.1. The computer program for the simulation was written in the freely available python programming language (version 2.5.1) for Windows (van Rossum, 2003).

Figure 4.3 shows the frequency distribution of the number of physical interactions between two consecutive spawning events. In this representative simulation, we have assumed that the underlying probability distribution of mating has the form $p(n) = 1 - e^{-0.1n}$. As we have seen previously, this distribution is diminishing in nature.

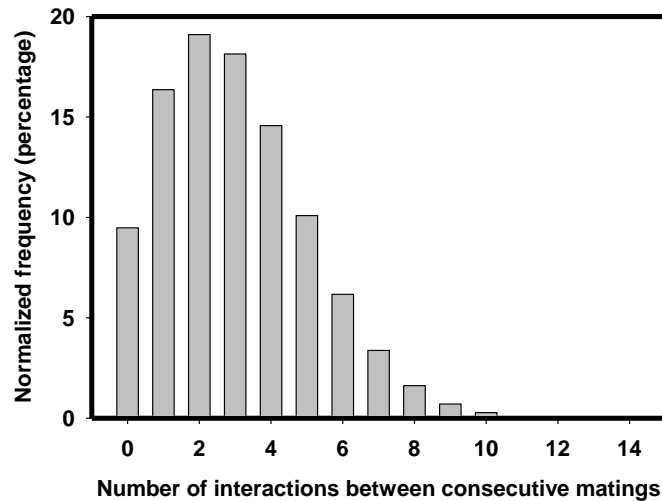


Figure 4.3

From Figure 4.3, it is clear that this frequency distribution, being discrete in nature, has the form of a Poisson distribution. The frequency distribution obtained from this representative simulation can, therefore, provide a baseline for comparing the experimental distribution and deciphering the extent of non-randomness in actual situations of spawning in zebrafish.

Figure 4.4 shows the return function (in the form of the probability that a female being courted will mate with the courting male) as a function of the number of physical interactions n between them. The return function is calculated from the frequency distribution obtained from the representative simulation according to the procedure outlined in the previous section. The figure

additionally plots the mating probability function fed into the program *a priori*. The close match between the calculated return function and the *a priori* probability distribution clearly shows that our method of calculating the return function is correct.

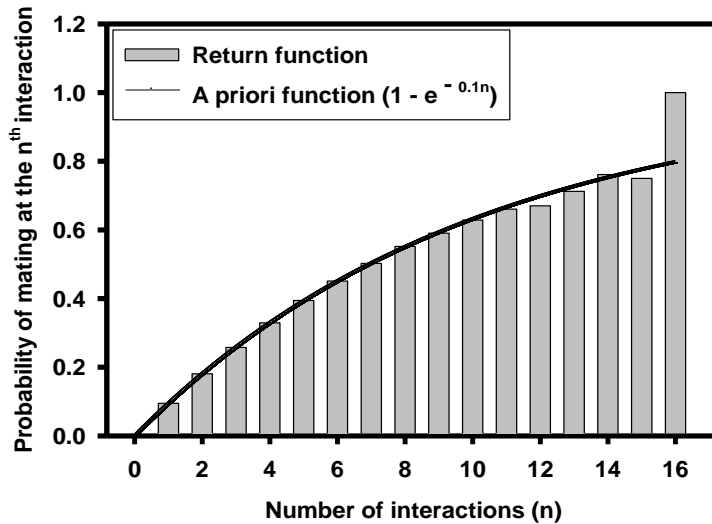


Figure 4.4

The probability of mating for the largest number of interactions (here 16) is seen to suddenly increase to 1. This apparent anomaly is actually an artifact of our attempt to calculate the return function (which has an infinite domain, extending from 0 up to infinity) from a finite dataset. As can be seen from the calculations described in the previous section, the return function $r(n)$ will always be equal to 1 at the largest possible value of n . The function essentially gets truncated at a finite value of n instead of extending up to infinity (where the probability tends to 1 in our assumed *a priori* probability function).

4.4 Experimental results

The frequency distribution of the number of male-female physical interactions between two consecutive spawning/mating events is shown in Figure 4.5 below. The frequency distribution initially does seem to increase just as in the case of the representative simulations – however, the number of observations are clearly too small to make any unambiguous conclusions about its shape and nature.

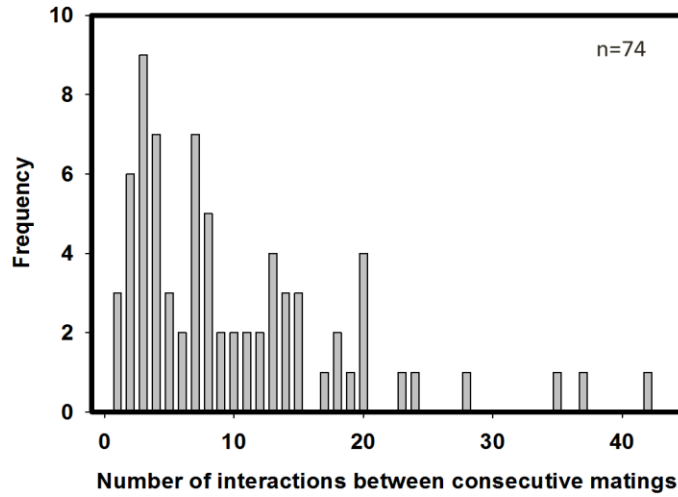


Figure 4.5

Figure 4.6 shows the return function calculated from this frequency distribution in the form of the mating probability at the n^{th} interaction. We can see that this function is clearly increasing in nature as expected – however, once again, the number of observations seem to be too small for us to be able to decipher its diminishing nature clearly. However, with probability being a bounded quantity (with its upper bound at 1), we can reasonably expect the increasing trend to distinctly display diminishing returns once we have more data from the experiments presently in continuation.

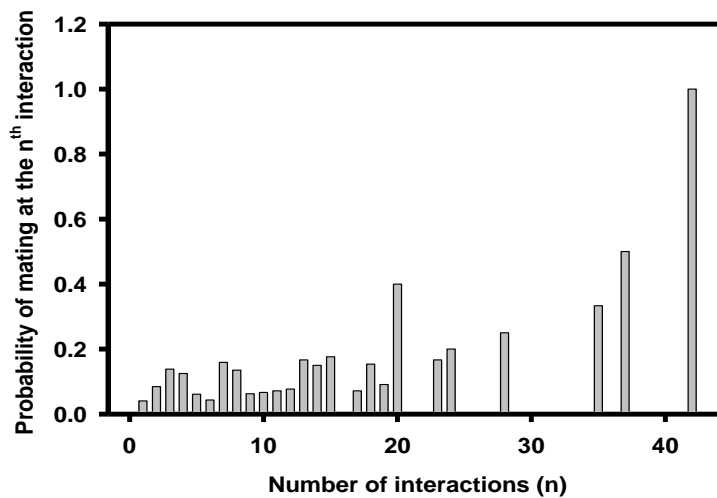


Figure 4.6

5. Conclusions and discussion

Optimality forms an over-arching concept in animal behavior. In the case of behavior involving repeated investments and diminishing marginal returns from them, Charnov (1976) showed optimal behavior attempts to maximize the rate of returns from these investments. The marginal value theorem, as it is called, then yields experimentally testable predictions about the optimal behavior.

In this study, we aimed to study courtship behavior in zebrafish *Danio rerio* under the optimality framework. The marginal value theorem, in this case, indicated to us that the time invested by a male in courting a female would be smaller if the male required less time to search for the female and *vice versa*. As it is clear that the time needed to search for a female would be smaller if the female density in the environment is high and *vice versa*, we predicted that the courtship time would be smaller when the female density is high than when it is low. Our experiments, which allowed a male physical access to only one female while simultaneously presenting him with visual, chemical, hormonal and olfactory cues from a variable number of other females unequivocally showed that the male spent significantly less time courting the physically accessible female when the total number of females giving him sensory cues was large and *vice versa* (Figure 3.5). Unlike other previous studies (Parker et al., 1993), our study of mating under the optimal behavior framework is uniquely able to separate out and subject courtship to the analysis of the marginal value theorem. It takes advantage of the fact that zebrafish is an externally fertilizing species – in this case, the actual length of the mating bout is extremely small and the variation in the time invested in courtship as a function of female density in the environment can be clearly tweezed out.

Though the support for the predictions of the marginal value theorem confirm the presence of diminishing marginal returns from repeated investments, we went on to attempt to construct the return function directly from experimental observations. In the case of courtship behavior in zebrafish, the return function would plot the probability that a female will mate with a courting male as a function of the time invested by the male in courtship. We, however, due to experimental limitations, decided to plot the returns as a function of the number of physical interactions (such as pushes and nudges) between the male and the female as a surrogate for the

time invested by the male in courtship. This was done with the understanding that a direct proportionality exists between the time invested by a male in courtship and the number of physical interactions between the male and the female. A representative simulation, with fishes assumed to be moving randomly like balls in a box showed that the frequency distribution of the number of physical interactions between two consecutive mating instances was Poisson in nature (Figure 4.3). The return function from the experimental observations appears to be increasing in nature – however, the number of observations in our data set presently seems to be insufficient to clearly indicate if the marginal returns are diminishing in nature (Figure 4.6).

This study, to our knowledge, is the only attempt to directly construct such a return function from experimental observations. In some sense, the return function, by plotting the probability that a female being courted will mate with the male courting it, reveals the female's sexual urge towards the male. Although the data set is small at the present juncture, our continuing observations can potentially provide enough data for us to make valid conclusions about the nature of a female zebrafish's sexual response to a male through the form of the return function.

It has to be kept in mind, however, that our attempt to construct the return function from experimental observations is based firmly on the assumption that a female's method of estimating its probability of mating with the male does not change significantly with time. In other words, we have assumed that the form and nature of the return function does not vary greatly between different sets of males and females, and does not change enormously from one mating event to the next. For instance, this would mean that if the return function is diminishing in nature, it is of this form for all the females we observed. It is this assumption which allows us to pool the data of physical interactions from replicate sets of observations and construct the frequency distribution of the form shown in Figure 4.5. This assumption seems quite reasonable since the return function is an inherent property of courtship behavior in zebrafish which is genetically determined and therefore, reasonably similar across individuals isolated from the same geographical area and mating events. It is, therefore, highly unlikely that there will be enormous variance in the way in which repeated investments of time in courtship from a male's side is interpreted by a female giving rise of very different forms of the return function across the observed individuals and with time.

It is, at this juncture, important to understand how an optimal mating and courtship strategy, according to the marginal value theorem, yields itself to adaptive evolution by maximizing the fitness of the individual adopting it. It is clear that the ultimate goal of courtship behavior on the part of the male is to produce a successful mating/spawning event. The reproductive fitness of a male is well accepted to be maximized by the maximization of the number of successful mating events he can participate in (Bateman, 1948). Therefore, the form of courtship which maximizes the overall probability of successful mating (or equivalently, the total number of mating events that a male participates in) is the one which is optimal in the sense of maximizing reproductive fitness and is selected for. The marginal value theorem predicts that optimal courtship behavior will dictate that the male spends less time courting each female if there are a large number of females available to him – in a sense, a male, under conditions of high female density, is susceptible to stop courting a female and go out in search for a new mate much earlier than if the female density in the environment is low. For any environment, we can calculate the total amount of resources available – in our case, this is equivalent to the number of females present, with the females being bearers of the resources (eggs) which the males aim to fertilize. It is now clear that the amount of resources available in an environment is much higher if the female density is high. In such a scenario, we can find out an average rate of returns through the utilization of resources (in the form of the probability of the occurrence of a successful mating) as a male invests time in courtship – this will be much lower in the situation with a smaller number of females being present. Therefore, even though the individual return function ($r(n)$ in our studies) may not change across different females, the marginal returns from increased time investment in courtship on the part of the male become smaller than the average returns for the entire environment much later if the number of females present is small (and hence, the average returns are small too). Following on the inference that the behavior which confers maximum reproductive fitness to a male will maximize the *overall* probability of successful mating, we expect that such a behavior will ordain that a male persists to court the same female as long as the marginal returns from the courtship are higher than the average returns from the environment (returns are in the form of the mating probability, as has been throughout this study). It immediately becomes clear that such a behavior will result in a male investing more time to court a female when the female density is low and *vice-versa* as in such an environment, the marginal returns from courting an individual female will go below the average returns for the environment

much later just because the returns from the environment, *on an average*, are low. It is now abundantly clear, with this complimentary understanding, that the optimal behavior as described by the marginal value theorem is simultaneously the one which maximizes the *average* probability of a successful mating, and thereby, the male's reproductive fitness. This is exactly identical to Charnov's (1976) alternative interpretation of the marginal value theorem wherein he inferred that an environment with a smaller number of patches of food will have a lower average rate of returns than one in which patches of food are more numerous. The marginal returns from the utilization of single patch by a foraging animal will become less than the average returns much later when the number of patches in the environment is small – the forager is then, according to the marginal value theorem, expected to spend more time foraging at each patch of food. A more generalized form of such an interpretation in the context of emigration thresholds is expanded upon in Parker and Stuart, 1976.

In essence, in this study, we aimed to investigate courtship behavior in zebrafish and attempted to see if the marginal returns from repeated investments of time by a male in courtship are diminishing in nature as required by the marginal value theorem. The results from our experimental studies offer strong support to optimal behavior being shaped by the marginal value theorem. Additionally, the trends observed in our attempt to measure the returns from repeated investments of a male's time in courtship deem it very possible that diminishing marginal returns from these investments will be distinguished once a large enough data set has been gathered.

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A.1. Python code to simulate random mating in zebrafish

Our simulation assumed fishes to be moving randomly like balls in a box. Any collisions (when the fishes would approach within a certain threshold distance of each other) were taken as physical interactions between them. The *a priori* mating probability function was assumed to be $p(n) = 1 - e^{-0.1n}$ where n is the number of physical interactions between the fishes. Additionally, this simulation is a two-dimensional abstraction of the realistic behavior of fishes – in this case, the fishes move randomly on a plane. The simulation also assumes periodic boundary conditions – essentially, each time a fish hits the boundary of the arena during its random motion, it reappears at the opposite boundary.

```
from random import *
from math import *

#Output file
g=open('B:/MSproject/Simulations/Random/test.out','w')
#Defining arena parameters
l=20.0
h=12.0

#Defining the mating probability function:
def feeling(n):
    value=1-exp(-0.1*n)
    return value

#Initiating fishes
f=[]
m=[]
f.append(l*random())
f.append(h*random())
m.append(l*random())
m.append(h*random())

#Time parameters
t=0
dt=1

#Step parameter
ds=1.0

#Distance parameter
```

```

d=0.0

#Interaction parameters
n=0
dthreshold=0.5

#Probability parameter
p=0.0

#Time evolution
while t<=10000000000:
    #Choosing random direction
    ftheta=2*pi*random()
    mtheta=2*pi*random()

    #Updating coordinates
    (f[0],f[1])=(f[0]+ds*cos(ftheta),f[1]+ds*sin(ftheta))
    (m[0],m[1])=(m[0]+ds*cos(mtheta),m[1]+ds*sin(mtheta))

    #Checking if boundary is hit
    #right
    if f[0]>1:
        f[0]=f[0]-1
    if m[0]>1:
        m[0]=m[0]-1
    #left
    if f[0]<0:
        f[0]=f[0]+1
    if m[0]<0:
        m[0]=m[0]+1
    #top
    if f[1]>h:
        f[1]=f[1]-h
    if m[1]>h:
        m[1]=m[1]-h
    #bottom
    if f[1]<0:
        f[1]=f[1]+h
    if m[1]<0:
        m[1]=m[1]+h

    #Calculating distance
    d=sqrt((f[0]-m[0])**2+(f[1]-m[1])**2)

    #Checking for collision
    if d<dthreshold:
        n=n+1
        #Monte Carlo for mating
        p=feeling(n)

```

```
        if p>=random():
            print>>g,n
            n=0 #Assuming that independent matings are separate
stochastic processes

        t=t+dt

g.close()
```