AN ANALYSIS OF INTRAOVARIAN SPERM INTERACTIONS IN THE
GUPPY, POECILIA RETICULATA

by

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We accept this thesis as conforming
to the required standard

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May 1980
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Abstract

Although guppy females may store sperm in the ovary for several months, they become sexually receptive for a short time every 22-30 days. Females may remate during these receptive periods and consequently, a potential competition between stored and fresh sperm for oocytes may occur. Earlier studies indicated that sperm from the most recent insemination dominates fertilization of new broods. However, these studies provide no clues to how this apparent sperm precedence occurs.

There are three possible explanations. First, sperm from new inseminations may outnumber stored sperm and thus, have a statistical advantage. Second, since fertilization in guppies occurs within a few days of insemination, this timing of insemination (relative to egg maturity) may give fresh sperm an advantage. Finally, the location of fresh sperm in the ovary may give them greater access to mature eggs.

In this study, females were artificially inseminated with known numbers of spermatophores to see how 1.) the proportion of one male's sperm in a mixed insemination determines its contribution to new broods; 2.) the number of spermatophores in inseminations affects the degree of precedence shown by sperm from re-inseminations; and 3.) the timing of re-insemination relative to the female reproductive cycle affects the degree of precedence shown by
re-inseminations. An autosomal, recessive allele was used as a genetic marker to distinguish between different males' sperm. In addition, ovaries of females artificially inseminated twice were examined histologically to locate sperm from each insemination. Sperm from one of the inseminations was radioactively labelled.

These experiments indicated that sperm precedence does not necessarily result from a statistical advantage to sperm from a re-insemination. Also sperm from re-inseminations occurring up to a week before a new batch of oocytes is mature have strong precedence over stored sperm, although the degree of precedence may be reduced. Autoradiographical analysis of sections of ovaries, however, indicated that the location of sperm storage sites may contribute to sperm precedence. Sperm clusters found in tubules closely associated with oocytes come predominantly from re-inseminations. A model is proposed suggesting that movement of sperm into these tubules immediately after insemination accounts for the dominance of new inseminations during fertilization. The possible functional significance of sperm storage and sperm precedence in poeciliids is discussed.
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Introduction

Prolonged sperm retention within female reproductive systems is a surprisingly common phenomenon throughout the animal kingdom. In keeping with the diversity of reproductive modes in animals, this sperm storage is highly variable. Both the degree and means of storing sperm vary between, and within, major taxonomic groups. Despite this variability, two basic problems are common to sperm storage in animals. First, since sperm have limited metabolic machinery, they have been considered incapable of long-term maintenance. The fact that sperm are known to survive in the female reproductive system for months or even years implies sophisticated physiological mechanisms for sperm storage. These mechanisms have been the subject of considerable inquiry (Forbes, 1961; Parker, 1970; Parkes, 1960; Thibault and Levasseur, 1973).

The second, and perhaps more important problem, is the significance of sperm storage as a reproductive strategy. The part that sperm storage plays in reproduction, how it enhances reproductive output, and the effect it has upon other aspects of reproduction are important for understanding the evolutionary and ecological consequences of sperm storage. In contrast to studies on the physiology of sperm storage, the evolutionary significance of sperm storage is exceedingly difficult to determine, and so has received little attention.
With the exception of the insects, sperm storage in the invertebrates is not as well known as it is in the vertebrates. In many invertebrates (e.g., opistobranch gastropods, turbellarians, arthropods), females possess structures to hold sperm before fertilization (Barnes, 1974) and these may be capable of maintaining sperm for prolonged periods. Unfortunately, the duration of sperm viability within these structures is unknown.

More is known about sperm storage in insects. Sperm storage is documented in five insect orders and is particularly well known in *Drosophila*. Parker (1970) reviewed the mechanisms employed by insects known to store sperm and considered the consequences and behavioural phenomena linked with sperm storage.

For the vertebrates a more extensive survey of female sperm storage is available. Every major vertebrate group contains members in which females store sperm and the diversity of adaptations is high. The viability of stored sperm ranges from a few days to years and, depending upon the group, storage sites can be folds, tubules, or diverticulae anywhere along the female reproductive tract. The relationship between sperm and female tissues ranges from a very simple association to one involving complex modifications of the storage site epithelium (Dent, 1970; Hoffmann and Wimsatt, 1972; Wimsatt et al., 1966). For a few groups, the anatomical details are well studied, but little is known about the physiology of sperm survival and activity during storage. Forbes (1961), Parkes (1960), and Thibault
and Levasseur (1973) reviewed sperm storage in vertebrates, but provided only a cursory treatment of the phenomenon in fish. Gardiner (1976) presents a more detailed review of fish.

Variability in the modes of storing sperm among the vertebrates suggests that sperm storage has different roles and consequences in vertebrate reproduction. Essentially two basic patterns of sperm storage occur in the vertebrates. Several groups delay fertilization for a prolonged period after copulation (bats, Wimsatt et al., 1966; some lizards and snakes, Fox, 1956, 1963; embiotocid fishes, Gardiner, 1976; blue shark, Pratt, 1979). In these animals, sperm storage is essential to reproduction. Presumably delaying fertilization ensures that copulation, gestation, and rearing occur only at optimal times of the year. In other groups fertilization occurs soon after copulation, but spermatozoa remain viable in the female reproductive system for considerable lengths of time. The purpose of sperm storage in such situation is not obvious and may vary from animal to animal. Perhaps it serves to maintain female reproductive output when male availability is low.

As a result of prolonged sperm storage in some vertebrates, viable sperm may be present in the female reproductive system when another insemination occurs. If this happens, there is a potential for competition for mature oocytes between the sperm of two males. The consequences of this are twofold. Depending on the nature
of the competition, female reproductive output may be affected in some way. For example, genetic variability within and between broods will depend upon the outcome of any intraspecific sperm interactions. In addition, an implicit competition between males may exist. Again, depending upon the outcome of sperm interactions, male success may be affected by a female's reproductive history.

Of the vertebrates, the poeciliids, a family of teleost fish, best exemplify the superimposition of multiple inseminations on prolonged sperm storage. In this group, reproduction is extremely specialized and includes: internal fertilization; either viviparity or ovoviviparity; sometimes superfoetation; and a well developed ability to store sperm in the ovary. A short and regular reproductive cycle also prevails in the group and is what makes sperm storage such a curious phenomenon in the poeciliids. Females are receptive to males for a short time in each cycle. This receptive period corresponds to the completion of oogenesis and fertilization. Viable sperm remain in the ovary through several cycles (van Oordt, 1928; Winge, 1937), and consequently females commonly retain the sperm of more than one male (Borowsky and Kallman 1976; Hildemann and Wagner, 1954; Winge, 1937).

Preliminary assessments of the success of sperm from different inseminations have been made. Breeding experiments using genetic markers indicate that in the guppy, *Poecilia reticulata*, an insemination directly following the birth of a brood has precedence in fertilizing
the next brood (Hildemann and Wagner, 1954; Winge, 1937). Since Winge (1937) does not support his claims with experimental results, it is impossible to assess his conclusions, but Hildemann and Wagner (1954) give the mating records of ten females. In eight, the re-insemination contributed almost entirely to the subsequent brood.

The significance of sperm storage in the reproductive biology of Poecilia reticulata cannot be fully understood until the factors governing sperm precedence are known. From the above studies it is clear that a second insemination predominates over the first in a subsequent brood, but since the quality and quantity of spermatozoa in these inseminations is unknown, little can be inferred about how a recent insemination gains precedence. The simplest explanation of sperm precedence is a disparity in the number of available spermatozoa between the two inseminations. It is expected that losses through fertilization and mortalities before and during sperm storage will reduce the number of stored sperm to levels below that of a new insemination. As a consequence, a male's success after a mating would depend upon the number of sperm in his insemination and the number of inseminations preceding his.

Many other hypotheses are also possible. Since re-insemination occurs when oocytes are mature (Rosenthal, 1952; Thibault and Schultz, 1978; Turner, 1937), sperm from a re-insemination may be in a position to fertilize oocytes before stored spermatozoa can. Alternatively, a competitive interaction between spermatozoa from different males or
differences in sperm viability, could explain the phenomenon. Clearly, an explanation of the mechanism of sperm precedence will help in understanding the importance of sperm storage in guppy reproduction, and also give insights into the physiology of sperm storage.

In the present study predictions based on two hypotheses about the factors governing sperm precedence are tested. I propose that if sperm precedence by the second insemination results from a disparity in the number of spermatozoa from the first and second inseminations, then the size of the inseminations should determine the disparity and hence the degree of sperm precedence. If the precedence gained by the second insemination results from insemination occurring at the same time eggs mature, changing the timing of the second insemination with respect to the female reproductive cycle should change the success of the second insemination. In addition I have employed autoradiography to determine the activity of sperm from two different inseminations within the ovary and their affect upon each other. Possibly, the location of stored sperm from two inseminations determines the amount of sperm precedence gained by re-inseminations.
General Methods and Materials

The basic technique used in this study was to artificially inseminate female guppies twice with known amounts of sperm, and then assess the interaction between the sperm from the two inseminations. To separate the results of the two inseminations, it was necessary that sperm from one insemination carried a marker. Where the success of an insemination was assessed through offspring paternity, a genetic marker was used. To identify the sperm of two different inseminations within the ovary, a radioisotope was used.

Stocks used in this study

The genetics of a large number of traits in Poecilia reticulata are known and so a variety of genetic markers are available. Unfortunately most are not easily obtained from aquarium supply outlets. For this study, I chose the xanthic mutant phenotype, "Gold", as a genetic marker. It is controlled by a single recessive autosomal allele (gd) (Goodrich et al, 1944; Winge, 1927) and gives the homozygote (gd/gd) a gold body colour.

Using the gd allele as a genetic marker has several advantages. Stocks are readily obtained from aquarium supply dealers. Also, newborn gd/gd individuals are easily
distinguished from both +/qd and +/+ young. This removes the need to raise the broods before scoring their phenotypes. Finally, the genetics of the trait are simple enough that phenotype frequencies directly indicate the frequencies of the two alleles in the broods.

Brood stocks of female and male qd/qd and +/+ guppies were initially obtained from Hartz Mountain Pet Supplies Limited. The two strains were maintained separately in 84 litre glass aquaria. Pregnant females were isolated in 20 litre glass aquaria until they had given birth and then were placed back into the appropriate brood stock tank.

Offspring of the two strains were kept separate in 40 and 84 litre glass aquaria. Since a stock of virgin females was required for the experiments, males were removed from the tanks as soon as their sex could be determined. They were kept in 40 litre glass aquaria and used as a sperm supply. Virgin females were not used in the experiments until they were at least four months old.

For the duration of the experiments, all fish were kept in an environment chamber at a constant temperature of 25°C and a constant photoperiod of 16 hours of light and 8 hours of darkness. All 20 litre aquaria were fitted with undergravel filters and a sand substrate. Filtration in the 40 and 84 litre aquaria was accomplished either with undergravel filters and a sand substrate or small corner filters.
Artificial insemination procedure.

To ensure that females were inseminated with known amounts of sperm, all inseminations were artificial. In essence, artificial insemination involves extracting spermatophores from a male, suspending these spermatophores in a saline solution, drawing the suspension into a micro-pipette, and injecting it into the female reproductive tract. The use of artificial insemination in studies of poeciliid genetics and reproduction is not new. Clark (1950) first described a simple procedure for inseminating Xiphophorus helleri. In Clark's technique, spermatophores were simply suspended in a 0.8% NaCl solution, and then, injected into the female. Billard (1966) described a similar technique for artificially inseminating Poecilia reticulata. However, since Billard inseminated female guppies with known amounts of sperm, his technique was more complex than Clark's. Billard suspended spermatophores in Flickinger's solution to preserve spermatophore integrity and measured the number of spermatophores in his inseminations volumetrically with a specially designed pipette.

My study also required known numbers of spermatophores in inseminations. However, rather than measure quantities of spermatophores volumetrically, I counted spermatophores. Not only did counting spermatophores give a more accurate measure of spermatophore number, but it also made it easier to mix spermatophores from different males.
a. **Obtaining sperm from males.**

Before stripping spermatophores from a male guppy, the male was anaesthetized in a 300 mg/l solution of MS 222. The time it took for the anaesthetic to have effect varied, but I usually waited just until the male stopped breathing. Guppies are extremely hardy and only a few males died from this treatment.

Once anaesthetized, the male was placed on a well-slide so that the base of the gonopodium was just inside the well. I then viewed the gonopodium under a dissecting microscope. The well of the well-slide contained a 0.8% NaCl solution and served as a collecting vessel for spermatophores. Holding the anterior half of the male with thumb and forefinger of one hand, its gonopodium was swung forward (Plate 1A) and held in place by slightly rotating the male so that the gonopodium was pressed against the slide. With a blunt dissecting needle, the testicular region, just anterior to the anus, was repeatedly stroked posteriorly until spermatophores stopped flowing into the well of the well-slide. When no more spermatophores were available, the male was carefully removed from the slide and placed in fresh aquarium water to recover from the anaesthetic. The well of the well-slide was then topped up with the 0.8% NaCl solution. Since males that recovered from the anaesthetic before the stripping was complete became impossible to handle, this stripping procedure had to be done quickly.

Although Billard (1966) found that spermatophores
dissociated in a 0.8% NaCl solution, in my artificial inseminations, spermatophores usually remained intact in 0.8% NaCl. Possibly, sperm survive in some salines better than in others, but unfortunately, this has not been investigated for guppy sperm. I used the NaCl solution since it was easy to prepare.

b.) Insemination of the female.

It was essential to work through this portion of the procedure as quickly as possible. Although spermatophores generally remained intact in 0.8% NaCl solution for up to ten minutes, sometimes they began dissolving soon after being removed from the male. When this occurred, the spermatophores either dissolved before they could be counted or they stuck to the glassware.

Once spermatophores were collected in the well of the well-slide, the desired number of spermatophores was counted. First, a micro-pipette was fashioned by heating and drawing out a Fisherbrand micro-hematocrit tube (1.2 mm I.D.) and breaking the tapered end to produce an opening approximately 0.5 mm in diameter. This micro-pipette was then attached with a piece of Intramedic polyethylene tubing (0.965 mm I.D.) to a 1 cc plastic syringe (Plate 1B). By slowly withdrawing the plunger of the syringe, spermatophores were drawn into the micro-pipette and counted one at a time.
When the required number of spermatophores were drawn into the micro-pipette, the micro-pipette was detached from the syringe and held vertically so that the spermatophores would settle into the tapered end. Occasionally, light tapping of the micro-pipette was necessary before the spermatophores would settle. Once the spermatophores had settled, they were transferred to a modified micro-capillary tube. This tube was fashioned by heating and drawing out one end of a 10 µl Drummond micro-capillary tube, breaking the tip of the tapered end, and then, fire polishing the broken tip. The diameter of the opening at the tapered end of the tube was approximately 0.35 mm (Plate 1C). Spermatophores in saline were transferred by capillary action to the micro-capillary tube by quickly touching the tapered ends of the micro-pipette and the micro-capillary tube. With practice it was possible to transfer the spermatophores in 2 to 3 µl of saline solution.

Next, the micro-capillary tube, containing spermatophores, was held vertically so that the spermatophores again settled to the tapered end. The tube was then lightly tapped to dislodge spermatophores sticking to the side. As soon as the spermatophores were settled the tube was set aside until a female was ready to be inseminated.

Females were anaesthetized the same way males were and then placed on their backs onto a piece of damp cheese cloth. Again it was necessary to do the insemination with the aid of a dissecting microscope. The micro-capillary
tube was attached to the rubber dispensor bulb supplied with the tubes and inserted, tapered end first, into the female reproductive duct (Plate 1D). The spermatophores and saline were slowly ejected from the micro-capillary tube by squeezing the bulb. As soon as the tube was evacuated, it was quickly withdrawn and the female was placed in fresh aquarium water to recover from the anaesthetic.

Aside from the obvious advantage of the micro-capillary being small enough to fit the reproductive tract of female guppies, it offered several other advantages. Since the volume of the tube was very small, the volume of fluid drawn into the micro-capillary tube, and the injection of spermatophores into the female, could be precisely controlled. Drawing the spermatophores into the micro-capillary tube by capillary action rather than by suction also meant more precise control in transferring spermatophores from the micro-pipette to the micro-capillary tube.

I attempted to keep the volume of spermatophore suspension to a minimum to prevent damage to the female and to avoid washing the spermatophores back out of the reproductive tract, but 2 μl was the smallest volume that could be achieved. Occasionally, females were inseminated with spermatophores in approximately 5 μl of saline. However, this did not appear to affect female survival nor the success of the insemination.
Plate 1:  

A) Male guppy with gonopodium partially erected.

B) Syringe, polyethylene tubing, and modified micro-hematocrit tube for counting spermatophores.

C) Modified micro-capillary tube with tapered end. The white clump at the tip of the tube is a clump of spermatophores.

D) Introduction of the micro-capillary tube into the female reproductive duct.
Section I

The relationship between the proportion of genetically distinct sperm in mixed inseminations and the relative contribution of those sperm to broods.

Introduction

Hildemann and Wagner (1954) and Winge (1937) investigated intraspecific sperm competition in Poecilia reticulata. In their studies females were mated and then, after the first brood, remated and the paternity of subsequent broods determined. Winge (1937) did not describe his mating procedure, but in all Hildemann's and Wagner's trials, females homozygous for xanthic, mutant, autosomal, recessive alleles were first mated with males carrying the same alleles and then remated with males homozygous for a dominant allele (usually the + allele). As a consequence of their experimental design, it is not possible to determine if the precedence of the second insemination is due to the sequence of inseminations carrying the two alleles or to the fact that the second insemination was made with sperm carrying a dominant allele at the marker locus. Perhaps the genotype of the second insemination determined its success. One of the aims of my study is to establish whether or not allelic differences in sperm affect fertilization success.

A second aim of this portion of the study is to establish the relationship between the proportion of a
male's sperm in the total ovary sperm pool and his contribution to a brood. My first hypothesis states that the reduction in the number of sperm from the first insemination which occurs between inseminations gives sperm from a later insemination precedence. A necessary corollary of this hypothesis is that when sperm from two males are used in the same insemination, the relative success of the two males' sperm is a function of the numbers of each male's sperm.

The experiments in this portion of the study are designed to answer both questions. By artificially inseminating females with varying proportions of $gd$ and $+$ sperm, not only have I simulated simultaneous inseminations by two males, but I can also assess the effect of male genotype on sperm success.

Methods and Materials

All inseminations in this section were artificial. Each insemination contained the spermatophores of both $gd/gd$ and $+/+$ males. Spermatophores were obtained from each male and kept separate in well-slides. Using a syringe, the appropriate number of spermatophores from each male were drawn into a micro-pipette (produced from a hematocrit tube) and the entire insemination transferred to a 10 µl micro-capillary tube. Each contained a total of 100 spermatophores, but with mixtures of 25:75, 50:50, and 75:25 of $gd : +$ spermatophores.
Virgin $gd/gd$ females, were inseminated once with one of the sperm mixtures. Twenty six inseminations were mixed 25:75; 26 inseminations were mixed 50:50; and 18 inseminations were mixed 75:25. These females were then isolated in 20 litre glass aquaria.

The young from consecutive broods were collected as soon after birth as possible and counted and scored for phenotype. Since females normally give birth at night, the young were not removed from the female's tank until morning. It is possible that the females ate some of their young, but unless this predation is selective, it is unlikely that this effected the results. The young born during the day appeared alert with a well developed escape response, and at no time did I observe a female eat any of her offspring. In an attempt to prevent females cannibalizing their offspring, I tried using breeding traps, but females did not do well in them. Using plants in the tank to provide the young with cover only proved to be a hindrance when removing young. Once the young were scored, the two phenotypes were kept separately in 40 litre glass aquaria and used for future experiments.

**Results**

Table 1 summarizes the results of the three sets of artificial inseminations. I have presented the number of young of each phenotype in all the first broods and then
Table 1: The number of progeny of each genotype in the first broods of females inseminated with three different proportions of gd sperm.
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<td>3</td>
<td></td>
</tr>
<tr>
<td>7.</td>
<td>1</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>8.</td>
<td>8</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>total</td>
<td>106</td>
<td>40</td>
<td></td>
</tr>
</tbody>
</table>
pooled offspring numbers to produce a total for each treatment group.

Table 1 indicates considerable variability in phenotype frequencies for all broods within each treatment group. This variability was highest when inseminations contained equal numbers of spermatophores from \( gd/gd \) and \( +/+ \) males. Females inseminated with other proportions of the two sperm types also produced broods with highly variable phenotype frequencies, but there was a clear tendency for the allele of the most abundant sperm in an insemination to predominate in each brood. A measure of heterogeneity in these data can be calculated using a G-statistic, but since brood sizes are small, its value is questionable.

As a result of small brood sizes, data in each treatment group were pooled and then compared to the expected phenotype frequencies (i.e., the three allelic frequencies in the inseminations). The \( +/gd \) frequency in broods from inseminations containing \( gd \) allele frequencies of .25, .50, and .75 are .27, .50, and .73. Table 2 presents a statistical comparison of the \( gd \) allele frequencies in the inseminations with the \( +/gd \) frequencies in the broods. The value of G for each treatment group is not significant at the 0.05 level.

Although 70 females were inseminated in this portion of the study, only 23 gave birth. Table 1 reports the percentage of successful artificial inseminations in each treatment group. These data suggest that inseminations with predominantly \( gd \) sperm were the most successful. Despite
Table 2: A comparison between the $gd/gd$ frequency in the broods and the $gd$ allele frequency in the inseminations for each treatment group.
<table>
<thead>
<tr>
<th>Proportion of gd/gd progeny in pooled broods</th>
<th>Proportion of gd allele in the inseminations</th>
<th>G (df = 1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.27</td>
<td>0.25</td>
<td>0.306</td>
</tr>
<tr>
<td>0.50</td>
<td>0.50</td>
<td>---</td>
</tr>
<tr>
<td>0.73</td>
<td>0.75</td>
<td>0.438</td>
</tr>
</tbody>
</table>

Values of G not significant at $p \leq 0.05$
this difference in artificial insemination success between treatment groups, pooled values in all treatment group are smaller than expected. Unfortunately, time and space did not permit me to re-do the experiment.

**Discussion**

The 1:1 relationship observed between the frequency of $gd$ allele in the inseminations and the "gold" phenotype frequency in the broods indicates that: 1.) allelic differences in sperm did not influence the results, and 2.) when the effects of a time lag between two inseminations were removed, male success was determined by the proportion of his sperm in the sperm pool. Both conclusions are important because they satisfy the conditions necessary for using the $gd$ allele as a genetic marker and for proposing further hypotheses.

Although the relationship is clear when broods within treatment groups are pooled, the phenotype frequencies in each brood are highly variable. This is probably in part due to the small size of most broods. Small differences in the number of each phenotype between small broods translate into substantial differences in the proportion of each phenotype. Unfortunately it is unlikely that larger broods can be obtained. Winge (1927) noted that with virgin females the first brood tended to be small and this appears to be the case in this, and my other, experiments.

Undoubtedly the artificial insemination technique is
another source of variability in brood phenotype frequencies. Spermatophores could not be obtained from the males simultaneously, and as a result, spermatophores from one male were suspended in saline solution longer than those of the other male. This may have produced differences in the viability of sperm from the two males. In addition, it was technically difficult to mix the spermatophores from the two males before an insemination. Because of these two factors, the males' sperm may not have been distributed randomly in the insemination, and thus fertilization by the different sperm may not have been random. Studies on the genetics of other alleles in *Poecilia reticulata* show phenotype frequency variability between individual crosses (Winge, 1927). Unfortunately, pooled data were used to establish the genetics of the gd allele (Goodrich et al., 1944) and therefore an independent measure of phenotype frequency variability between individual broods after natural inseminations is unavailable. Since the problem of small brood sizes is compounded by errors introduced by artificial insemination, analysis of differences between broods would be difficult to interpret.

The importance of establishing the effect of sperm genotype on sperm success in this study cannot be overlooked. It is entirely possible that in competitive situations, small allelic differences may confer differential success upon sperm. An example is the xanthic mutant phenotype, "Cream", in *Poecilia reticulata*. "Cream" is controlled by recessive, autosomal alleles at two loci.
and has been used as a genetic marker in mating studies (Haskins and Haskins, 1950; Hildemann and Wagner, 1954). This phenotype, however, characteristically shows reduced viability and consequently the sperm carrying the allele or the resulting hybrid zygotes may be less viable than normal genotypes. Prout and Bungaard (1977) have shown that simple genetic differences in *Drosophila melanogaster* can affect sperm success in competitive situations.

Of the 70 females inseminated, only 23 gave birth. This low success indicates that artificial insemination is not completely effective. Other workers (Billard, 1966; Clark, 1950; Liley, pers. comm.) have also reported low success with artificial inseminations in poeciliids.

Low success with artificial insemination may be the result of two factors. Since spermatophores were transmitted in a physiological saline solution, the composition of the solution may have affected sperm viability. Clark (1950) used a 0.8% NaCl solution and reported 60% success with *Xiphophorus*, another genus of poeciliid. To maintain the integrity of spermatophores while inseminating guppies, Billard (1966) used Flickinger physiological saline and reported levels of success comparable to Clark's. Gardiner (1976) used a buffered saline to study sperm storage in *Cymatogaster aggregata*. He indicated that pH was critical to sperm survival. The actual insemination may also have contributed to the low success. Insertion of a micro-pipette into the female reproductive system may damage the system or in some other
way upset the female's reproductive physiology.

In addition to these possible disadvantages in artificial insemination, the reproductive condition of the females may be responsible for some of the lack of insemination success. Liley (1968) postulated an endogenous reproductive cycle controlling egg maturation and egg degeneration in virgin females which had a period of approximately 30 days (similar to the period of the non-virgin female brood cycle). If such a reproductive cycle occurs in virgin females, inseminations when mature eggs are beginning to degenerate and the next batch of oocytes are beginning to mature would mean delayed fertilization for one reproductive cycle (i.e. if these females were capable of storing sperm) and subsequent delay in the production of a brood. Thus, I may have discarded some females before they gave birth. However, I cannot exclude the possibility that some females in this experiment were infertile.

The difference in insemination success between treatment groups suggests that \( gd \) and \( + \) sperm may differ in viability or vigor. This suggestion contradicts the brood phenotype frequency data from each treatment group, but the fact that insemination success was greatest when inseminations contained predominantly \( gd \) spermatophores appears to indicate that \( gd \) sperm are more vigorous than \( + \) sperm. The relation between insemination success and the proportion of \( gd \) sperm in inseminations also contradicts data in the experiments in the following sections. The data in these experiments suggest that \( + \) sperm may be more
vigorous than gd sperm. This inconsistency in the relative successes of gd and + sperm is discussed in the next section. Although refinement of the artificial insemination technique may increase its effectiveness, most likely, large numbers of inseminations would have to be performed to generate moderate sample sizes. In this study, doubling or tripling the number of inseminations would have required much more time and space than was available.
Section II  The effect of spermatophore number in inseminations on the success of re-inseminations.

Introduction

Little can be inferred from previous studies of intraspecific sperm competition in guppies (Hildemann and Wagner, 1954; Winge, 1937) about the nature of interactions between sperm from different inseminations or the phenomenon sperm precedence. In these studies, males and females were kept together for up to twenty days. Under such circumstances it is impossible to estimate both the amount of sperm introduced at each insemination and the number of inseminations performed by each male. Consequently the influence of the amount of sperm introduced by a male on that male's success is not known.

The observation that a second insemination has precedence in fertilizing eggs over the first insemination can be explained by a disparity in the number of sperm available for fertilizing eggs from each insemination. Sperm from the second insemination probably outnumber sperm from the first insemination for a number of reasons, but the most obvious is a reduction through either fertilization of an earlier brood or storage of only a portion of the sperm from the first insemination. The direct relationship observed in the previous section between the proportional
representation of a male's sperm in the total sperm pool and his success in contributing to a brood makes the sperm number hypothesis appear reasonable. A number of artificial insemination experiments were designed to test the hypothesis.

The hypothesis predicts that when the size of the first and second inseminations are equal, any disparity between the number of sperm from each insemination available to fertilize the second brood will be due to a reduction in the number of sperm from the first insemination. Note, however, that there may be threshold effects. If the size of inseminations are so large that the ovary is "saturated" with sperm, the second insemination may not have precedence simply because enough sperm still remain from the first insemination to do an effective job. The purpose of the present section is to test this prediction. The procedure is similar to that of Hildemann and Wagner (1954), but since the inseminations are artificial the amount of sperm is known.

Methods and Materials

Three groups of virgin gold females were artificially inseminated with 40, 100, and 200 spermatophores. These quantities were chosen to produce a large range in the size of the ovary sperm pool. Billard (1966) demonstrated that the quantity of sperm in an insemination affects the number of offspring per brood and the number of broods a female
produces. In his study females inseminated with 40 or fewer spermatophores produced successively smaller broods. Successive broods produced after inseminations with 100 spermatophores, on the other hand, were larger and did not significantly change in size. These observations indicate that the amount of stored sperm from inseminations containing 100 spermatophores does not diminish as noticeably as it does for smaller inseminations. After doubling the size of Billard's largest insemination, I assumed sperm levels in the ovary were sufficiently high to reduce the difference in the number of first and second insemination sperm available for fertilization of the second brood.

Each female was kept in a 20 litre glass aquarium until she gave birth to her first brood. The young were collected from the aquarium as soon after birth as possible, counted, and then either discarded, or if they were the gold phenotype, saved for future use. The female was then immediately re-inseminated (the second insemination) with the same amount of sperm as used in the first insemination.

Since females usually give birth at night, re-insemination usually did not immediately follow birth. Although this time lapse between birth and re-insemination could not be accurately known, it never exceeded 24 hours. The period of time between birth and the fertilization of the next brood has been reported to be from six to twelve days (Dildine, 1936; Rosenthal, 1952; Turner, 1937). If this is so, re-insemination in my experiments occurred well
before completion of fertilization of the next brood. Females were returned to their tanks after re-insemination and the offspring of the second broods were counted and their paternity determined.

To determine how much each insemination contributed to the second brood, one of the inseminations carried the \( gd \) allele. Within the three treatment groups, some of the females received the genetically marked sperm in the first insemination and others received the marked sperm in the second insemination. This was done to control for the possibility that the sperm genotype affects sperm success. The schedule of inseminations and number of inseminations are presented in Table 3.

Not all females were inseminated a second time. Some died before parturition, and others (either because of infertility or a poor insemination) did not give birth. Since the average time between the first artificial insemination and the first brood was 36 days, females that did not give birth within 50 days were discarded from the experiment.

Results

Again I experienced difficulty obtaining an adequate sample size by using artificial insemination. Table 3 presents the number of females initially inseminated, the
Table 3: The number of females inseminated at the beginning of the experiment, the number of females re-inseminated, and the number re-inseminated females that gave birth a second time. Values for the two insemination sequences in each treatment group are separated.
<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Insemination sequence</th>
<th>No. of females inseminated</th>
<th>No. of females re-inseminated</th>
<th>No. of re-inseminated females giving birth</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (insem. with 40 spermatophores)</td>
<td>gd;+</td>
<td>26</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>+;gd</td>
<td>21</td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td>B (insem. with 100 spermatophores)</td>
<td>gd;+</td>
<td>27</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>+;gd</td>
<td>17</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>C (insem. with 200 spermatophores)</td>
<td>gd;+</td>
<td>24</td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>+;gd</td>
<td>24</td>
<td>13</td>
<td>6</td>
</tr>
</tbody>
</table>
number of females which gave birth and were re-inseminated, and the number of females which gave birth after the second insemination. Table 4 gives the percentage of successful inseminations. The overall success of artificial insemination was low, but success varied between some groups of inseminations. Insemination of virgin females with gd sperm tended to be less successful than insemination with + sperm. However, the success of these inseminations with gd sperm did increase as the number of spermatophores in inseminations increased. The success of the first insemination with + sperm also increased slightly as the number of spermatophores in inseminations increased.

The success of second inseminations was greater than the success of first inseminations. In addition, the success of inseminations with gd or + sperm was similar. This difference in success between first and second inseminations may simply be the result of selection for fertile females. The virgin females used in this experiment may not all have been fertile, but since only females which had given birth were re-inseminated, only fertile females received the second insemination.

Table 5 presents the number of gd/gd and +/gd offspring in first and second broods of all females successfully inseminated twice. The order of insemination with gd and + sperm, as well as the number of spermatophores used in inseminations, are also indicated. In all cases, second inseminations had precedence over first inseminations in contributing to the second brood. The degree of precedence
Table 4: The percentage of successful inseminations and re-inseminations for each insemination sequence in the three treatment groups.
<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Insemination sequence</th>
<th>% of total females re-inseminated</th>
<th>% of re-inseminations resulting in birth</th>
<th>% of total females giving birth after re-insemination</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (insem. with 40 spermatothophores)</td>
<td>gd;+</td>
<td>19.2</td>
<td>60.0</td>
<td>11.5</td>
</tr>
<tr>
<td></td>
<td>+;gd</td>
<td>42.8</td>
<td>66.7</td>
<td>28.6</td>
</tr>
<tr>
<td>B (insem. with 100 spermatothophores)</td>
<td>gd;+</td>
<td>25.9</td>
<td>57.1</td>
<td>14.8</td>
</tr>
<tr>
<td></td>
<td>+;gd</td>
<td>47.1</td>
<td>62.5</td>
<td>29.4</td>
</tr>
<tr>
<td>C (insem. with 200 spermatothophores)</td>
<td>gd;+</td>
<td>37.5</td>
<td>66.7</td>
<td>25.0</td>
</tr>
<tr>
<td></td>
<td>+;gd</td>
<td>54.2</td>
<td>46.2</td>
<td>25.0</td>
</tr>
</tbody>
</table>
Table 5: The number of \(+/gd\) and \(gd/gd\) progeny in first and second broods produced by females in the three treatment groups.
<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Female</th>
<th>Insemination sequence</th>
<th>No. of gd/gd progeny</th>
<th>No. of +/gd progeny</th>
<th>No. of gd/gd progeny</th>
<th>No. of +/gd progeny</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (insem. with 40 spermatophores)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.</td>
<td></td>
<td>gd;+</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>43</td>
</tr>
<tr>
<td>2.</td>
<td></td>
<td>gd;+</td>
<td>25</td>
<td>-</td>
<td>4</td>
<td>45</td>
</tr>
<tr>
<td>3.</td>
<td></td>
<td>gd;+</td>
<td>12</td>
<td>-</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>4.</td>
<td></td>
<td>+;gd</td>
<td>-</td>
<td>17</td>
<td>36</td>
<td>1</td>
</tr>
<tr>
<td>5.</td>
<td></td>
<td>+;gd</td>
<td>-</td>
<td>33</td>
<td>36</td>
<td>-</td>
</tr>
<tr>
<td>6.</td>
<td></td>
<td>+;gd</td>
<td>-</td>
<td>15</td>
<td>36</td>
<td>-</td>
</tr>
<tr>
<td>7.</td>
<td></td>
<td>+;gd</td>
<td>-</td>
<td>12</td>
<td>39</td>
<td>-</td>
</tr>
<tr>
<td>8.</td>
<td></td>
<td>+;gd</td>
<td>-</td>
<td>26</td>
<td>8</td>
<td>3</td>
</tr>
<tr>
<td>9.</td>
<td></td>
<td>+;gd</td>
<td>-</td>
<td>32</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>B (insem. with 100 spermatophores)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.</td>
<td></td>
<td>gd;+</td>
<td>11</td>
<td>-</td>
<td>-</td>
<td>27</td>
</tr>
<tr>
<td>2.</td>
<td></td>
<td>gd;+</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>43</td>
</tr>
<tr>
<td>3.</td>
<td></td>
<td>gd;+</td>
<td>12</td>
<td>-</td>
<td>-</td>
<td>30</td>
</tr>
<tr>
<td>4.</td>
<td></td>
<td>gd;+</td>
<td>12</td>
<td>-</td>
<td>-</td>
<td>49</td>
</tr>
<tr>
<td>5.</td>
<td></td>
<td>+;gd</td>
<td>-</td>
<td>8</td>
<td>30</td>
<td>-</td>
</tr>
<tr>
<td>6.</td>
<td></td>
<td>+;gd</td>
<td>-</td>
<td>26</td>
<td>14</td>
<td>2</td>
</tr>
<tr>
<td>7.</td>
<td></td>
<td>+;gd</td>
<td>-</td>
<td>20</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td>8.</td>
<td></td>
<td>+;gd</td>
<td>-</td>
<td>44</td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td>9.</td>
<td></td>
<td>+;gd</td>
<td>-</td>
<td>1</td>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td>C (insem. with 200 spermatophores)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.</td>
<td></td>
<td>gd;+</td>
<td>43</td>
<td>-</td>
<td>2</td>
<td>47</td>
</tr>
<tr>
<td>2.</td>
<td></td>
<td>gd;+</td>
<td>21</td>
<td>-</td>
<td>9</td>
<td>22</td>
</tr>
<tr>
<td>3.</td>
<td></td>
<td>gd;+</td>
<td>14</td>
<td>-</td>
<td>5</td>
<td>36</td>
</tr>
<tr>
<td>4.</td>
<td></td>
<td>gd;+</td>
<td>59</td>
<td>-</td>
<td>6</td>
<td>62</td>
</tr>
<tr>
<td>5.</td>
<td></td>
<td>gd;+</td>
<td>32</td>
<td>-</td>
<td>-</td>
<td>60</td>
</tr>
<tr>
<td>6.</td>
<td></td>
<td>gd;+</td>
<td>41</td>
<td>-</td>
<td>-</td>
<td>61</td>
</tr>
<tr>
<td>7.</td>
<td></td>
<td>+;gd</td>
<td>-</td>
<td>31</td>
<td>42</td>
<td>-</td>
</tr>
<tr>
<td>8.</td>
<td></td>
<td>+;gd</td>
<td>-</td>
<td>25</td>
<td>38</td>
<td>2</td>
</tr>
<tr>
<td>9.</td>
<td></td>
<td>+;gd</td>
<td>-</td>
<td>25</td>
<td>14</td>
<td>1</td>
</tr>
<tr>
<td>10.</td>
<td></td>
<td>+;gd</td>
<td>-</td>
<td>31</td>
<td>24</td>
<td>1</td>
</tr>
<tr>
<td>11.</td>
<td></td>
<td>+;gd</td>
<td>-</td>
<td>13</td>
<td>15</td>
<td>-</td>
</tr>
<tr>
<td>12.</td>
<td></td>
<td>+;gd</td>
<td>-</td>
<td>5</td>
<td>31</td>
<td>-</td>
</tr>
</tbody>
</table>
varied considerably, but in half of the cases sperm from second inseminations contributed to 100% of the second brood.

Table 6 indicates two trends in the mean size of first and second broods in all treatments. First, second broods in all treatments tended to be larger than first broods. Second, the mean size of first and second broods tended to increase as the number of spermatophores in the inseminations increased. A consistent trend in brood sizes did not occur between treatments A and B (inseminations with 40 and 100 spermatophores), but a clear increase in the mean size of first and second broods occurred after inseminations with 200 spermatophores.

In addition to the differences in mean brood size between treatment groups and first and second broods, broods produced after inseminations with gd sperm appeared to differ in size from broods produced after inseminations with + sperm. Table 6 also gives the mean size of broods produced after inseminations with gd and + sperm. When comparing mean size of first and second broods, note that females inseminated first with gd sperm were re-inseminated with + sperm and vice versa.

The mean brood sizes reported in this table indicate that broods produced after inseminations with gd sperm tended to be smaller than broods produced after inseminations with + sperm. Figure 1A and 1B illustrate how mean brood size varied between sperm genotype and treatment group in first and second broods. In Figure 1A, the mean
Table 6: The mean sizes of first and second broods resulting from inseminations with either gd or + sperm. The mean sizes of pooled broods for each treatment group are also reported. The value in parentheses is the standard deviation and n is the number of broods.
<table>
<thead>
<tr>
<th>No. of spermatophores in the inseminations</th>
<th>First Broods</th>
<th>Second Broods</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Insem. with \textit{gd} sperm</td>
<td>Insem. with + sperm</td>
</tr>
<tr>
<td>40</td>
<td>13.0 (11.5) \textit{n = 3}</td>
<td>22.5 (9.1) \textit{n = 6}</td>
</tr>
<tr>
<td>100</td>
<td>9.2 (4.9) \textit{n = 4}</td>
<td>19.8 (16.7) \textit{n = 5}</td>
</tr>
<tr>
<td>200</td>
<td>35.0 (16.3) \textit{n = 6}</td>
<td>21.7 (14.8) \textit{n = 6}</td>
</tr>
</tbody>
</table>
Figure 1A: Mean size of first broods after inseminations with 40, 100, and 200 spermatophores. Bars represent 1 standard deviation on either side of the mean.

Figure 1B: Mean size of second broods after inseminations with 40, 100, and 200 spermatophores. Bars represent 1 standard deviation on either side of the mean.
size of first broods produced after inseminations with + sperm remained constant as the number of spermatophores in the inseminations increased. In contrast, mean brood size remained low after inseminations with 40 and 100 gd spermatophores, but after inseminations with 200 gd spermatophores, the mean brood size increased markedly. Figure 1B indicates that the mean size of second broods also tended to be largest after inseminations with + sperm. Note that, in addition to this, the mean size of second broods increased as the number of spermatophores in the inseminations increased.

It appears from these mean brood size data that gd sperm are not as vigorous or viable as + sperm. However, the sudden increase in the mean size of first broods following insemination with 200 gd spermatophores (Fig. 1A) suggests that the number of gd sperm in an insemination may have to reach a threshold level before they are as effective as + sperm. It is difficult to speculate further with these few data. Since a similar trend in the mean size of second broods (Fig. 1B) did not occur after inseminations with gd sperm, possibly the trend seen in Figure 1A is an artifact of variable data and small sample size. Table 7 presents results of an analysis of variance comparing brood size variability among treatments, order of insemination with gd and + sperm, and first and second broods. Among these categories, the differences were significant at the 0.05 level.

To test the null hypothesis that the number of
Table 7: An analysis of variance comparing brood sizes among insemination sequence, first and second broods, and treatment groups.
<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>SS</th>
<th>MS</th>
<th>F test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>59</td>
<td>15613.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Among insemination sequences</td>
<td>1</td>
<td>1337.9</td>
<td>1337.9</td>
<td>7.86*</td>
</tr>
<tr>
<td>Among broods</td>
<td>1</td>
<td>1771.3</td>
<td>1771.3</td>
<td>10.40*</td>
</tr>
<tr>
<td>Among treatment groups</td>
<td>2</td>
<td>2064.4</td>
<td>1032.2</td>
<td>6.06*</td>
</tr>
<tr>
<td>Interaction</td>
<td>7</td>
<td>2267.5</td>
<td>323.9</td>
<td>1.90</td>
</tr>
<tr>
<td>Within</td>
<td>48</td>
<td>8172.3</td>
<td>170.3</td>
<td></td>
</tr>
</tbody>
</table>

* significant at the 0.05 level
spermatophores in inseminations does not affect the success of sperm in re-inseminations, I compared the relative frequencies of each phenotype in the second brood between treatment groups. Since, the second insemination was always the most successful and the number of females in each of the groups was small, I pooled the data for each treatment group before making the comparison. The results of the comparison using a chi-squared contingency test for three independant samples are presented in Table 8. With a chi-square equal to 0.852, df=2, no significant difference in phenotype frequency exists between the second broods of each of the three treatment groups. Consequently the null hypothesis cannot be rejected and I conclude that the amount of sperm in inseminations does not effect the success of the second insemination.

Discussion

The simplest hypothesis explaining the apparent advantage that second inseminations have over first inseminations is a statistical one. In all fertilizations a proportion of the available sperm will be used. If fertilization efficiency is not high, the number of sperm lost in this way may be considerable. As a result, the number of sperm left in the ovary after fertilization will be less than in the original insemination. If the next
Table 8: Contingency table for a chi-square test comparing the contribution of first and second inseminations to second broods between the three treatment groups. Values in parentheses are calculated expected values.
<table>
<thead>
<tr>
<th>Treatment group</th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of progeny produced from the first insemination.</td>
<td>11</td>
<td>14</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>(13.7)</td>
<td>(12.3)</td>
<td>(25.0)</td>
</tr>
<tr>
<td>Total number of progeny produced from the second insemination.</td>
<td>251</td>
<td>222</td>
<td>452</td>
</tr>
<tr>
<td></td>
<td>(248.3)</td>
<td>(223.7)</td>
<td>(453.0)</td>
</tr>
</tbody>
</table>

chi-square = 0.852: not significant at p ≤ 0.05, 2df
insemination is as large as the first, and if fertilization is a stochastic process, then the second insemination will be at a statistical advantage in fertilizing the next brood of eggs. Depending upon the number of sperm in each insemination and the fertilization efficiency of each insemination, the success of the second insemination may vary.

It is clear from the results of my artificial insemination experiments that the prediction is not borne out. There is no difference in the pooled phenotype frequencies of the broods across treatment groups. In all broods, the genotype of the second insemination predominates. This indicates that a difference in the number of sperm from two inseminations is not necessarily a cause of the apparent advantage of the second insemination.

Possibly 200 spermatophores were not enough for any disparity between the number of stored sperm (from the first insemination) and the number of fresh sperm (from the second insemination) to be markedly different from a disparity in sperm number resulting from inseminations with 40 spermatophores. This, however, is unlikely. Billard's (1966) observation that the size of consecutive broods dwindles after one insemination with 50 spermatophores, but remains constant after one insemination with 100 spermatophores, suggests that loss of sperm due to fertilization and storage is more noticeable after inseminations with less than 50 spermatophores than after inseminations with more than 100 spermatophores. Therefore,
if sperm from the second insemination had precedence during fertilization simply because they outnumbered sperm from the first insemination, re-inseminations with 200 spermatophores should have had the least success in my experiments. This was not the case.

The amount of precedence shown by second inseminations in this experiment further strengthens the argument that this precedence cannot be accounted for by a purely statistical hypothesis. If sperm from re-inseminations gained precedence because they outnumbered stored sperm, the strong dominance of second inseminations in fertilizing second broods would have meant that 95% of the sperm from first inseminations were lost. Billard's (1966) data, however, suggest that much less sperm is lost after an insemination. If 95% of the sperm in an insemination were lost, consecutive broods produced after inseminations with 100 spermatophores would dwindle in size as rapidly as broods produced by females inseminated with 40 spermatophores rather than remaining constant.

On the basis of differences in brood size between first and second broods, one could argue that sperm from second inseminations predominate in fertilization of second broods because these sperm are more vigorous. Brood size data in this experiment indicate that females produced larger second broods than first broods and this may suggest greater vigor or viability in sperm from second inseminations. However, Rosenthal (1952), Turner (1937), and Winge (1922) noted that first broods produced by females inseminated once were also
the smallest. Since fecundity is positively correlated with female size in *Poecilia reticulata* (Purser, 1938; Thibault and Schultz, 1978; Turner, 1937), they suggested that this difference between the sizes of first and second broods resulted from an increase in fecundity with growth. Although in my experiment, I did not measure a female's length each time she gave birth, most females were noticeably smaller at the birth of their first brood than they were at the completion of the experiment. Therefore, the difference in mean brood size between first and second broods was probably due to fecundity increasing with age and growth.

Although sperm from the second insemination dominated in fertilizing the second brood, in all cases, artificial insemination with gd sperm tended to be less successful than artificial insemination with + sperm. Both the number of successful inseminations and the size of resulting broods were less when gd sperm were used. These data suggest that gd sperm may be less viable or vigorous than + sperm. However, as I mentioned in Section I, differences in artificial insemination success, and brood size, associated with differences in sperm genotype are not consistent. The brood phenotype frequencies obtained in the experiment in Section I indicate there is no difference in vigor between + and gd sperm, but differences in the success of artificial insemination between different treatments in the experiment suggest that gd rather than + sperm may be the most vigorous.
Factors such as fecundity and female fertility also affect brood size and success of insemination. Since the number of females in each treatment group of my experiments was small, any factors (other than sperm genotype) which could affect brood size and insemination success may not have varied randomly among the different groups of inseminations. Therefore, although these differences appear to be related to sperm genotype differences, they may be fortuitous. On the other hand, phenotype frequencies within broods directly reflect different sperm viabilities. Since mean phenotype frequencies in the experiments of this section did not differ between inseminations with $gd$ sperm and inseminations with $+$ sperm, $gd$ and $+$ sperm are probably equally vigorous.

Although a difference in the number of sperm available from first and second inseminations is not necessary to give second inseminations precedence, this does not mean that differences in the size of two inseminations do not affect fertilization success in subsequent broods. The size of inseminations probably varies from male to male, and if a female was re-inseminated with a smaller amount of sperm than in the preceding insemination, the second insemination may not fare well. A similar situation may exist if a female has been inseminated several times. The last insemination may be so small compared to the amount of sperm stored in the ovary that its success is reduced. The outcome of such situations cannot be determined from the results of this part of my study; however, if two unequal
inseminations were performed in a set of inseminations similar to the ones in this part of the study, or the success of a third insemination was monitored, further insight into the problem may be gained.

Several other hypothesis can account for second inseminations gaining precedence over first inseminations. Stored sperm, either because of their age or because of a lack of some vital substance present during insemination, may be less viable than sperm from fresh inseminations. Alternatively, stored sperm may be in regions of the ovary that give them less access to mature eggs than sperm from a new insemination or they may be displaced from storage sites by fresh sperm. Some of these hypothesis are not as difficult as others to test experimentally and they should be investigated first. The next section looks at the effect of time of re-insemination relative to the female brood cycle on the degree of precedence gained by the second insemination. If some characteristic of storage makes stored sperm less successful than fresh sperm, a new insemination well before eggs are mature (hence sperm from both inseminations are stored) may not have precedence over the first insemination during fertilization of freshly matured eggs.
Section III The effect of timing of re-insemination on the success of sperm from re-inseminations.

Introduction

Under natural conditions the timing of insemination is restricted in poeciliids. Breeding is continuous in many populations, but individual females are receptive for only a few days after parturition (Carlson, 1969; Liley, 1966). In most poeciliids, this period of receptivity corresponds to the final maturation of the next batch of eggs. Consequently, new inseminations introduce fresh sperm into the ovary just as a new set of eggs become available for fertilization.

In all previous studies of intraspecific sperm competition in *Poecilia reticulata*, inseminations were natural (Bowden, 1970; Hildemann and Wagner, 1954; Winge, 1937). Since this occurs only when females are receptive and carrying mature eggs, it has been impossible to determine the influence that the timing of an insemination, relative to egg maturation, has on fertilization. Since mature eggs are available at the time a new insemination, or just after insemination, freshly introduced sperm may have greater access to eggs than stored sperm. Sperm activity before fertilization has not been described, so it is not possible to compare the relative success of competing stored sperm and fresh sperm.
In this section, I use artificial insemination to examine the effects of the time of re-insemination on success. Females were re-inseminated during gestation and the success of this insemination is compared to that of re-inseminations occurring directly after parturition (the natural time of re-insemination).

Methods and Materials

51 virgin gdm/qd females were artificially inseminated with 100 spermatophores. 27 of the inseminations were with gdm sperm and 24 with + sperm. Approximately 7 to 12 days later all females were re-inseminated with 100 spermatophores, but of the genotype opposite to that used in the first insemination. Females were then kept separately in 20 litre glass aquaria.

The time between the two inseminations was chosen so that the re-inseminations occurred after fertilization of the first batch of eggs, but before development of the second brood proceeded far. In this way, only the first insemination could contribute to the first brood, and the possibility of physical damage to the developing embryos from the re-insemination was minimized. Although Stolk (1950) reported that an occlusion apparatus closes the gonoduct opening during brood development, I was able to insert the micro-capillary tube containing spermatophores into the female tract with little difficulty and no physical
damage to the female was apparent. 

Despite the lapse of 7 to 12 days between the initial insemination and re-insemination, the second insemination contributed to the first brood of some females. undoubtedly in virgin females the period between insemination and complete fertilization of the first brood is variable. An endogenous reproductive cycle controlling egg maturation has been postulated for virgin females (Liley, 1968). Possibly eggs were at various stages of maturity when I inseminated the virgin females in my experiments.

I used only those females whose first brood was fertilized entirely by sperm from the first insemination in the analysis. If the first brood was fertilized by a mixture of sperm from the two inseminations, this meant that re-insemination occurred prior to the beginning of embryo development and was not consistent with my experimental design. In addition, I could not be certain of the viability of sperm from the first insemination, if the brood was fertilized predominantly by sperm from the second insemination.

The success of the second insemination in this section is compared to that of treatment B of the previous section. This provides the appropriate comparison for determining how the timing of a re-insemination affects its success. The only difference between the two treatments is the time of re-insemination relative to the female reproductive cycle.
Results

Table 9 presents the number of $+/gd$ and $gd/gd$ progeny in the first and second broods of females in both treatments. The data for females re-inseminated post-partum (Treatment B) are from Table 5, Treatment B. In all cases, the second insemination contributed the most to the second brood. However, the amount of dominance gained by the second insemination differed between treatments and inseminations with different sperm genotypes.

The mean size of broods fertilized after inseminations with either $gd$ or $+$ sperm are given separately for each treatment group in Table 10. These data indicate two clear trends. First, second broods were larger than first broods in both treatments. Second, females in Treatment A produced larger broods (first and second broods) than females in Treatment B. An analysis of variance of brood sizes (Table 11) indicates that both of these brood size differences are significant for $p < 0.05$.

In contrast, differences in brood size between broods fertilized after inseminations with $gd$ sperm and broods fertilized after inseminations with $+$ sperm do not follow a consistent trend. Females in Treatment B tended to produce the largest broods after inseminations with $+$ sperm. In Treatment A, broods fertilized after inseminations with $gd$ sperm tended to be slightly larger than those broods fertilized after inseminations with $+$ sperm. The analysis of variance calculation in Table 11 indicates that the
Table 9: The number of $+/gd$ and $gd/gd$ progeny in first and second broods produced by females after pre- and post-partum re-inseminations.
<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Female</th>
<th>Insemination sequence</th>
<th>No. of gd/gd progeny</th>
<th>No. of +/gd progeny</th>
<th>No. of gd/gd progeny</th>
<th>No. of +/gd progeny</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
<td></td>
<td>First Brood</td>
<td></td>
<td>Second Brood</td>
<td></td>
</tr>
<tr>
<td>1.</td>
<td>gd;+</td>
<td></td>
<td>31</td>
<td>-</td>
<td>4</td>
<td>62</td>
</tr>
<tr>
<td>2.</td>
<td>gd;+</td>
<td></td>
<td>13</td>
<td>-</td>
<td>3</td>
<td>39</td>
</tr>
<tr>
<td>3.</td>
<td>gd;+</td>
<td></td>
<td>4</td>
<td>-</td>
<td>-</td>
<td>39</td>
</tr>
<tr>
<td>4.</td>
<td>+;gd</td>
<td></td>
<td>-</td>
<td>2</td>
<td>46</td>
<td>15</td>
</tr>
<tr>
<td>5.</td>
<td>+;gd</td>
<td></td>
<td>-</td>
<td>20</td>
<td>17</td>
<td>2</td>
</tr>
<tr>
<td>6.</td>
<td>+;gd</td>
<td></td>
<td>-</td>
<td>23</td>
<td>60</td>
<td>13</td>
</tr>
<tr>
<td>7.</td>
<td>+;gd</td>
<td></td>
<td>-</td>
<td>3</td>
<td>34</td>
<td>16</td>
</tr>
<tr>
<td>8.</td>
<td>+;gd</td>
<td></td>
<td>-</td>
<td>14</td>
<td>45</td>
<td>-</td>
</tr>
<tr>
<td>B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>First Brood</td>
<td></td>
</tr>
<tr>
<td>1.</td>
<td>gd;+</td>
<td></td>
<td>11</td>
<td>-</td>
<td>-</td>
<td>27</td>
</tr>
<tr>
<td>2.</td>
<td>gd;+</td>
<td></td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>43</td>
</tr>
<tr>
<td>3.</td>
<td>gd;+</td>
<td></td>
<td>12</td>
<td>-</td>
<td>-</td>
<td>30</td>
</tr>
<tr>
<td>4.</td>
<td>gd;+</td>
<td></td>
<td>12</td>
<td>-</td>
<td>-</td>
<td>49</td>
</tr>
<tr>
<td>5.</td>
<td>+;gd</td>
<td></td>
<td>-</td>
<td>8</td>
<td>30</td>
<td>-</td>
</tr>
<tr>
<td>6.</td>
<td>+;gd</td>
<td></td>
<td>-</td>
<td>26</td>
<td>14</td>
<td>2</td>
</tr>
<tr>
<td>7.</td>
<td>+;gd</td>
<td></td>
<td>-</td>
<td>20</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td>8.</td>
<td>+;gd</td>
<td></td>
<td>-</td>
<td>44</td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td>9.</td>
<td>+;gd</td>
<td></td>
<td>-</td>
<td>1</td>
<td>10</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 10: The mean sizes of first and second broods resulting from inseminations with either gd or + sperm. The mean sizes of pooled broods for each treatment group are also reported. Values in parentheses are the standard deviations and n is the number of broods.
<table>
<thead>
<tr>
<th></th>
<th>Insem. with gd sperm</th>
<th>Insem. with + sperm</th>
<th>Pooled broods</th>
<th>Insem. with gd sperm</th>
<th>Insem. with + sperm</th>
<th>Pooled broods</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pre-partum re-inseminations</strong></td>
<td>16.0 (13.8)</td>
<td>12.4 (9.6)</td>
<td>13.8 (10.5)</td>
<td>49.6 (20.2)</td>
<td>47.7 (12.5)</td>
<td>49.4 (17.2)</td>
</tr>
<tr>
<td>n</td>
<td>3</td>
<td>5</td>
<td>8</td>
<td>5</td>
<td>3</td>
<td>8</td>
</tr>
<tr>
<td><strong>Post-partum re-inseminations</strong></td>
<td>9.3 (4.9)</td>
<td>19.8 (16.7)</td>
<td>15.1 (13.4)</td>
<td>17.4 (7.5)</td>
<td>37.3 (10.5)</td>
<td>26.2 (13.4)</td>
</tr>
<tr>
<td>n</td>
<td>4</td>
<td>5</td>
<td>9</td>
<td>5</td>
<td>4</td>
<td>9</td>
</tr>
</tbody>
</table>
sequence of inseminations with gd and + sperm (i.e. gd → + or + → gd) did not affect brood size trends between first and second broods and treatment groups. This means that brood size differences in this experiment are not related to sperm genotype differences.

Although brood size differences between treatment groups and first and second broods were not related to the sequence of inseminations with gd and + sperm, the degree of dominance shown by the second insemination appears to differ between the two insemination sequences. As seen in Table 9, there was a tendency for the second insemination to be more dominant when + rather than gd sperm were used in the insemination. Chi-square analysis of the available data (Tables 12A and 12B) shows this difference in dominance between inseminations with gd or + sperm to be significant (p < 0.05) in both Treatments A and B. Unfortunately the number of broods resulting from these inseminations may be too small to overcome the affects of factors other than sperm genotype which could bias the data.

Table 13 presents a chi-square contingency table of second brood phenotype frequencies in females subjected to pre- and post-partum re-inseminations. Despite the apparent frequency differences between re-inseminations with gd and + sperm, broods in each treatment group were pooled. At p < 0.05, df=1, the chi-square value, 8.67, indicates a significant difference in the degree of precedence gained between pre- and post-partum re-inseminations. Therefore the null hypothesis (that there is no difference in the
Table 11: An analysis of variance comparing mean brood sizes among insemination sequence, first and second broods, and treatment groups.
<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>SS</th>
<th>MS</th>
<th>F test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>33</td>
<td>12345.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Among insemination sequences</td>
<td>1</td>
<td>47.8</td>
<td>47.8</td>
<td>0.273</td>
</tr>
<tr>
<td>Among broods</td>
<td>1</td>
<td>4359.6</td>
<td>4359.6</td>
<td>24.86*</td>
</tr>
<tr>
<td>Among treatment groups</td>
<td>1</td>
<td>1510.6</td>
<td>1510.6</td>
<td>8.61*</td>
</tr>
<tr>
<td>Interaction</td>
<td>4</td>
<td>1868.3</td>
<td>467.1</td>
<td>2.66</td>
</tr>
<tr>
<td>Within</td>
<td>26</td>
<td>4560.0</td>
<td>175.4</td>
<td></td>
</tr>
</tbody>
</table>

* significant at the 0.05 level
Table 12: Contingency tables for chi-square tests comparing the contribution of first and second inseminations to second broods between insemination sequences for A) pre-partum re-inseminations and B) post-partum re-inseminations. Values in parentheses are calculated expected values.
A)  

<table>
<thead>
<tr>
<th>Insemination sequence</th>
<th>gd;+</th>
<th>+;gd</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of progeny produced from the first insemination.</td>
<td>7</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>(14.7)</td>
<td>(23.3)</td>
</tr>
<tr>
<td>Total number of progeny produced from the second insemination.</td>
<td>140</td>
<td>202</td>
</tr>
<tr>
<td></td>
<td>(132.3)</td>
<td>(209.7)</td>
</tr>
</tbody>
</table>

chi-square = 18.01: significant at p < 0.05, 1 df

B)  

<table>
<thead>
<tr>
<th>Insemination sequence</th>
<th>gd;+</th>
<th>+;gd</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of progeny produced from the first insemination.</td>
<td>0</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>(8.8)</td>
<td>(5.2)</td>
</tr>
<tr>
<td>Total number of progeny produced from the second insemination.</td>
<td>149</td>
<td>73</td>
</tr>
<tr>
<td></td>
<td>(140.2)</td>
<td>(81.8)</td>
</tr>
</tbody>
</table>

chi-square = 25.19: significant at p < 0.05, 1 df
Table 13: Contingency table for a chi-square test comparing the contribution of first and second inseminations to second broods between treatment groups. Values in parentheses are calculated expected values.
<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Total number of progeny produced from the first insemination.</th>
<th>Total number of progeny produced from the second insemination.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment group A</td>
<td>53 (42)</td>
<td>342 (353)</td>
</tr>
<tr>
<td>Treatment group B</td>
<td>14 (25)</td>
<td>222 (210)</td>
</tr>
</tbody>
</table>

chi-square = 8.67: significant at $p < 0.05$, 1df
success of pre- and post-partum re-inseminations) must be rejected.

Since I do not know whether the viability of sperm from first inseminations or the time of re-insemination relative to egg maturation affected contribution of re-inseminations to first broods in some females in Treatment A, it is impossible to compare the relative success of insemination and re-insemination between Treatments A and B. In addition, I was unable to determine the differences in success between artificial inseminations with $gd$ and artificial inseminations with $+$ sperm in Treatment A of this experiment.

Discussion

In the previous sections of this study, only differences in brood size, or artificial insemination success, appeared related to differences in sperm genotype. The competitive abilities of $gd$ and $+$ sperm were equal. In this experiment, however, differences in ability of sperm from re-inseminations to dominate during fertilization are correlated with sperm genotype, but differences in mean brood size are not associated with sperm genotype. This inconsistency in the association of brood differences and sperm success with sperm genotype suggests that $gd$ and $+$ sperm may be approximately equal in vigor and that stochastic processes and errors associated with technique may have biased the small samples of broods.
Rejection of the null hypothesis must be interpreted with care. Different degrees of sperm precedence associated with sperm genotype difference mean that after pooling broods, the amount that sperm from re-inseminations dominate fertilization will depend on how broods following re-insemination with gd and * sperm are represented in the pooled brood size. In Treatment B, broods fertilized after re-inseminations with * sperm were the largest and hence contributed the most to the pooled brood size. In Treatment A, more second broods were fertilized after re-inseminations with gd sperm and consequently they contributed the most to the pooled brood size in that treatment group. The result of this difference between Treatments A and B in the weighting of second broods fertilized after re-inseminations with the two sperm genotypes is that re-inseminations in Treatment A appear less able to dominate fertilization than re-inseminations in Treatment B.

Since there is doubt about whether or not sperm genotype affects the success of sperm in competition, the apparent difference in success between pre- and post-partum re-inseminations may be fortuitous. In addition, if factors other than sperm genotype affected brood size, or artificial insemination success, in a non-random way, the small sample size may have allowed these biases to be emphasized.

If sperm from pre-partum re-inseminations are indeed less capable than sperm from post-partum re-inseminations of dominating fertilization of second broods, it is not clear why this difference occurs. Possibly, developing embryos
occlude portions of the ovarian lumen and prevent sperm from reaching some of the storage sites. Also, the ovary's capacity to store sperm may be less during gestation. Unfortunately, anatomical evidence for these possibilities is lacking.

Despite differences in the degree of precedence gained by re-inseminations between treatment groups and sperm genotype in this experiment, sperm from all re-inseminations had precedence over sperm from the first insemination. Thus, although the time of re-insemination relative to the female reproductive cycle and sperm genotype may affect the success of re-inseminations, the second insemination still maintains precedence over the first insemination.

The results of my experiments up to this point indicate that:

1. the total sperm pool in the ovary after a female is re-inseminated does not work as a complete unit during fertilization. Instead sperm from recent inseminations are favoured.

2. dominance of sperm from second inseminations over sperm from first inseminations is established even before eggs are mature. The results of a few inseminations not reported here suggest that this dominance may be established immediately after re-insemination.

3. dominance is not always complete and the degree of dominance shown by sperm from re-inseminations may vary depending on circumstances.

It is, nevertheless, impossible to get a clear picture of
how sperm from re-inseminations dominate over sperm from first inseminations with this information alone. Direct indication of how sperm from different inseminations are distributed throughout the ovary is required as well. The purpose of the final part of this study was to gather some information about sperm distribution in the ovary and the results of that investigation are reported in the next section.
Section IV  Autoradiographical analysis of the location of sperm from separate inseminations in the ovary.

Introduction

My earlier experiments in this study demonstrated that sperm storage in the ovary must be organized since sperm from the most recent insemination are more likely to fertilize mature eggs than sperm from earlier inseminations. The disposition of sperm in the ovary may contribute to this advantage of new sperm over older sperm.

In this section, I investigate the anatomy of sperm storage by using sperm from two inseminations. A possible explanation for sperm precedence is that sperm from the most recent insemination are stored at locations that give clear access to mature eggs. Alternatively, if sperm move into all sperm storage sites after inseminations, sperm from new inseminations may displace older sperm from storage sites.

Previous workers (Bailey, 1933; Jalabert and Billard, 1969; Kadow, 1954; Ryder, 1885; Stuhlman, 1887; Winge, 1922) reported pockets and diverticulae of the ovarian lumen of guppies. Many of these diverticulae contact eggs and some workers suggested that these might be the site of fertilization (Billard, 1966; Jalabert and Billard, 1969). If sperm are normally stored in those diverticulae contacting eggs, this may be where dominance of sperm from new inseminations over sperm from earlier inseminations is
established. By using a radioisotope to mark sperm from different inseminations, it should be possible to determine if either of the two explanations of sperm precedence are tenable.

Methods and Materials

a.) Production of radioactively labelled sperm.

The autoradiographical procedure used in this study was developed to large extent by Luytens (unpubl. MS). 250 μCi of thymidine (methyl-^3H) (specific activity approximately 50 μCi/m mole) dissolved in 250 μl of sterile water was obtained from New England this concentration was used throughout the experiment. Labelled thymidine was administered by intraperitoneal injection with a 50 μl Hamilton syringe fitted with a 30 gauge needle. A male was anaesthetized with MS 222 and then placed on its side on a piece of dampened paper towel. The procedure was viewed with a dissecting microscope. Spermatophores were first stripped from the male in the manner described earlier. The needle was then pushed anteriorly under the integument dorsal to the base of the gonopodium and 2 μl of the undiluted thymidine solution were injected into the peritoneal space. The needle was quickly, but gently, withdrawn and the male was placed in fresh aquarium water to recover.
Five days after the first injection, males were re-injected on the opposite side of their bodies with 2 µl of the thymidine solution. Thus the total radioactive dose administered to males was 4 µCi. Luyten (unpubl. MS) found this dosage to result in the same amount of labelling as larger doses, but the volume of fluid used was less likely to cause harm to the male. After the injections, males were kept in 20 litre glass aquaria until the 28th day after the first injection. Tests by Luytens (unpubl. MS) indicated that labelling reaches a maximum (approximately 80%) 28 days after the first injection and then drops sharply a few days later.

b.) Artificial insemination of females.

The pattern of artificial inseminations was similar to that in the previous section: virgin females were inseminated twice with equal amounts of sperm and the time between inseminations was approximately a third of the reproductive cycle. To distinguish sperm from the two inseminations, sperm from one of the inseminations were labelled with tritiated thymidine. To reduce problems interpreting the autoradiographic data, inseminations were carried out in the following manner.

Three virgin females were initially inseminated artificially with 100 spermatophores. Ten days later they
were again inseminated, but this time with 100 spermatophores containing labelled sperm. In addition, two other virgin females were similarly inseminated, but the order of insemination with labelled and unlabelled sperm was reversed. All females were maintained in 20 litre glass aquaria until the tenth day after re-insemination.

Reciprocal inseminations were important for two reasons. First, they allowed me to assess differences in the activity of radioactively labelled and unlabelled sperm within the ovary. Secondly, this was the only means of determining if sperm from both inseminations occur together or separately in storage sites. Since radioactive sperm are detected indirectly on a photographic emulsion, radioactive sperm could mask the presence of unlabelled sperm in sperm clusters. Comparing the results of a reverse set of reciprocal inseminations would reveal whether this was occurring.

To check the radioactivity of labelled sperm, a set of virgin females was inseminated with 100 spermatophores from all of the radioactively treated males. Thus for every female inseminated with both labelled and unlabelled sperm, there was a control female inseminated with the same batch of labelled sperm. These control females were maintained in 20 litre glass aquaria and removed at the same time as the corresponding treatment female.
c.) **Histological procedures.**

Ten days after the second insemination, females (and corresponding control females) were killed for histological examination of the ovaries. Females were killed in Bouin's fixative and after approximately one hour, they were decapitated. This allowed penetration of the fixative into the internal organs. Fixation continued overnight.

After fixation the ovaries were excised from the bodies and washed in several changes of 70% ethanol. This part of the preparation was important for subsequent sectioning. Because of their high yolk content, fish eggs become hard from fixation. If any fixative remains, the eggs tend to shatter during sectioning, and thus destroy surrounding tissues.

Once the ovaries were washed, the tissues were dehydrated and infiltrated in a Fisher Histomatic Tissue Processor, Model 166. The ovaries were treated with two changes of 95% ethanol at two hours per change, and then treated with two changes of 100% ethanol, also at two hours per change. Final dehydration of tissues before Paraplast infiltration was done in two changes of xylene, the first change for twenty minutes, the second change for one hour. Shorter treatment times in xylene left the ovaries too hard for satisfactory sectioning. Infiltration of tissues with Paraplast was done in two changes of Paraplast at two hours per change. Infiltrated ovaries were finally embedded in Paraplast so that transverse sections could be made.
The ovaries were serially sectioned at 10 µm thickness. Although care was taken to preserve as many of the sections as possible, difficulties in sectioning as a result of egg hardness made it impossible to preserve all of the sections. Once mounted upon slides, sections were washed in xylene to remove the Paraplast and coated with a nuclear track emulsion.

d.) Application and development of the nuclear track emulsion.

Kodak NTB nuclear track emulsion was used to detect radioactively labelled sperm in tissue sections of ovaries. Like all nuclear track emulsions, this is very sensitive to light and therefore, the emulsion was applied to the slides and developed after exposure, in a darkroom, either in the dark or, if it was necessary to see, with a safety light.

The stock emulsion, normally stored at 4°C, was melted in a water bath at 45°C. This took approximately 30 to 40 minutes. Once melted, a small amount of the emulsion was diluted 1:1 with distilled water and placed in a clean, 50 ml glass vial. One by one, the glass slides containing tissue sections were held in the diluted emulsion for 2 seconds, pulled out, and the excess emulsion allowed to run back into the vial. Coated slides were stood upright and left to air dry in the dark for two hours.
Once dried, the slides were placed in slide boxes along with a package of Dryerite. The boxes were sealed with black electrical tape, wrapped in a heavy, black, plastic bag, and stored in the refrigerator at 4 C for 17 days. After 17 days of exposure, the slide boxes were opened in the darkroom using a safety light and the coated slides were processed in the following manner.

1. 4 minutes in D-19 Developer (at 20 C).
2. 30 seconds in water (at 20 C).
3. 8 minutes in Kodak fixer (at 20 C).
4. 1 minute in Edwal 4 and 1 hypoeliminator (at 20 C).
5. Washed in water for 20 minutes.
6. Air dried.

When the emulsion on the slides was dry, the tissue sections were stained with Ehrlich's hematoxylin and eosin stain and then permanently covered. Sections were examined under both bright field illumination and phase contrast with a Zeiss GFL microscope.

Results

The gross anatomy of the guppy ovary was described by Turner (1937). The ovary in *Poecilia reticulata* is a single, hollow organ which occupies a major portion of the body cavity. The ovarian lumen occupies mainly the posterodorsal part of the ovary, and connects to the exterior via a
short duct that exits just posterior to the anus. In the caudal portion of the ovary the oocytes are lateral and ventral to the lumen, but more anteriorly they restrict the lumen. Figure 2 presents a diagram of a longitudinal section of a guppy ovary.

The cross-sectional shape of the lumen varies between females, and can be anywhere from dorso-ventrally flattened to laterally flattened. In approximately the anterior two-thirds of the ovary, projections of the lumen extend amongst the oocytes. There is no general pattern to the distribution of these projections, but they are in close proximity to all oocytes. The epithelium enclosing the lumen is composed of a single layer of columnar cells.

Apparently oocyte distribution within the ovary is correlated with oocyte maturity. Three levels of oocyte development, as well as developing embryos, were evident in females inseminated twenty days before fixation. The smallest oocytes occurred centrally in the ovary, surrounding the lumen and its projections. These were distinguished from the other oocytes by a dense, homogeneous appearance (Plate 2B). Usually a small nucleus occurred in the center of each, but occasionally only a hole was present. Successive stages of egg development occurred more distal to the lumenal projections and embryos tended to occur towards the outside of the ovary. Oocytes at an intermediate stage of development possessed an obvious nucleus, surrounded by a vesicular-like matrix (most likely yolk). A cuboidal cellular layer surrounded the entire
Figure 2: Diagram of a longitudinal section of a guppy ovary. Details of internal structures are not to scale.
Plate 2: A) A tubule contacting an oocyte. Exposure of the emulsion can be seen at the distal end of the tubule.

B) An immature oocyte at an early stage of development. Nucleus is evident and is surrounded by a homogeneous matrix.

C) An immature oocyte at an intermediate stage of development. Note the intensely exposed area of the emulsion (lb) at the distal end of a tubule contacting the oocyte.

D) Magnification of Plate 2A showing spermatozoa and exposed emulsion at the distal end of the tubule.

E) Magnification of Plate 2C showing the intensely exposed area of the emulsion (lb). Spermatozoa are just visible at the edge of the exposed areas.

F) Same tubule as in Plate 2E, but one section later. The spermatozoa appear to be oriented with the tubule epithelium.

Symbols

eg - oocyte
lb - exposed emulsion
lm - ovarian lumen
sp - spermatozoa
tb - tubule
oocyte (Plate 2C). I could not see a nucleus in the largest oocytes, and although the egg consisted of a dense, homogeneous matrix (yolk), this matrix tended to be broken up. A layer of squamous cells surrounded oocytes at this stage of development.

Associated with each oocyte was a single tubule of varying length. These tubules apparently represent invaginations of the columnar layer of the lumenal epithelium. Mostly, the tubules end blindly, and separate the oocyte or follicular membrane of older oocytes from the lumen by a single columnar epithelial layer. Occasionally however, tubules appear to open onto the larger, yolky oocytes. The tubules also persist where embryos are developing, but here these tubules are completely open. Undoubtedly, young pass through these channels at birth. Plate 2A illustrates a tubule contacting an oocyte.

Apparently, sperm are stored in the tubules. In some cases, I found sperm associated with the epithelium of the main portion of the ovarian lumen, particularly with the dorsal epithelium; however, such sperm occurred in low numbers and without any consistent orientation to the epithelium. In contrast, sperm abounded in the tubules near the distal end. Such sperm were generally oriented in a regular pattern with the epithelium (Plate 2F). Unfortunately, the quality of tissue preparation did not allow a finer examination and I was unable to assess the actual association between the tubule epithelium and the sperm. Most frequently, sperm were in tubules associated
with the oldest oocytes, but sperm could be found in the tubules associated with oocytes of all ages.

Although the autoradiographic analysis of sperm activity was relatively successful, it was not as precise a diagnostic tool as I had hoped. I had hoped that radioactive levels in sperm would be high enough to identify individual, labelled sperm. This was not so. Radioactive sperm could only be detected when sperm were clumped and close to the surface of the tissue section. Since sperm generally occurred at high densities in tubules, finding labelled sperm there presented no problem, but distinguishing between labelled and unlabelled sperm was impossible. Ideally, a comparison of the relative proportion of marked sperm in the control and treatment females would give the most information. Unfortunately, high sperm densities and the indirect way of detecting radioactivity made sperm counts unreliable.

When females were inseminated with unlabelled sperm and then re-inseminated with labelled sperm, radioactivity was detected in all tubules that contained sperm. In tubules with high densities of sperm the label was particularly evident. At lower densities, signs of radioactivity were scattered. Unfortunately, I could not tell if all of these sperm were labelled. In the reciprocal set of inseminations radioactivity could not be detected, although control females contained radioactive sperm. Again, I could not tell if all sperm were from the second insemination since labelled sperm could have been present in low numbers, but
the indication from the two sets of inseminations is that sperm from the second insemination predominated in the tubules. The ovaries of treatment females were not examined if radioactive sperm could not be found in the ovaries of the corresponding control females. For this reason, one female from each of the reciprocal inseminations was discarded.

Plates 2D, 2E, and 2F show levels of radioactivity in clusters of sperm for two different inseminations. In Plate 2D, the female was inseminated initially with unlabelled sperm and then re-inseminated with labelled sperm. Radioactive labelling does not appear heavy in this photomicrograph, but at lower magnification (Plate 2A) greater exposure of the emulsion is evident. In Plates 2E and 2F, the female was re-inseminated with radioactively labelled sperm only. Note that the correlation between exposed emulsion and sperm is not perfect and that the extent of exposed emulsion may vary between sections. Possibly this difference in detection of label between sections results from sperm occurring at different depths in the section.

Jalabert and Billard (1969) and Kadow (1954) reported sperm in antero-dorsal diverticulae of the ovarian lumen and they considered these diverticulae to be the major sperm storage site. Although in my sections, the lumen of some ovaries ended anteriorly in blind pockets, I did not find sperm clusters in them. Occasionally sperm were present in these pockets, but never in large numbers. Thus, even if
some of these sperm were labelled, I probably would not have detected radioactivity.

Discussion

The ovary of the guppy is an extremely complex organ that probably serves several functions. Not only is it the site of oogenesis, but also it is the site of fertilization. In addition, it provides the necessary requirements for internal embryo development. In many poeciliids, only gas exchange and protection are provided by the ovary, but in other species the female provides some, or all, of the nutritional requirements (in addition to yolk) of the embryo (Scrimshaw, 1945; Thibault and Schultz, 1978; Turner, 1940). In contrast to other ovoviviparous or viviparous fish, the ovarian follicle plays an important role in ontogenesis in poeciliids. This structure remains intact until parturition (Turner, 1937).

This particular mode of ovoviviparity (and viviparity) can occur only in conjunction with special modifications for fertilization. If the oocyte does not enter the lumen before fertilization, there must be some way for sperm to penetrate the barriers between sperm and egg. In *Poecilia reticulata*, this problem apparently is solved by the contact between tubules and oocytes. The consistency and intimacy of the tubule and oocyte association suggests the tubules
are the site of fertilization.

Other workers also noted the existence of tubules. Ryder (1885), Stuhlman (1887), and Bailey (1933) referred to them as "delles". Winge (1927) and Kadow (1954) both reported sperm in the tubules, and Billard (1966) referred to them as "entonnoir de fécondation", implying that they function as a route for spermatozoa during fertilization.

The development of this association between tubules and oocytes has not been described. Apparently the association develops early, since tubules are found associated with the smallest oocytes in the ovary. Sperm do not appear to discriminate between tubules, and are found in tubules associated with oocytes of any age. Winge (1927) presents a photomicrograph of a cluster of sperm in a tubule contacting a small, immature oocyte.

There is some question, however, as to the exact site of sperm storage. Sperm commonly occur in the tubules for up to twenty days after insemination, but the duration of viability in these structures has not been assessed (Kadow, 1954; Winge, 1927). The presence of spermatozoa in antero-dorsal pockets of the lumen also is known (Jalabert and Billard, 1969; Kadow, 1954). Curiously, Jalabert and Billard (1969) do not mention sperm storage in the tubules. They consider the site of sperm storage to be in the alveolated diverticulae of the antero-dorsal portion of the ovary. Ultrastructurally, sperm in this region are embedded in folds of the epithelial cell plasma membranes, a situation common in vertebrates that store sperm.
Although in some females I found pockets at the antero-dorsal end of the ovarian lumen, these pockets contained few sperm. In addition, I occasionally found sperm in the dorsal epithelium of the lumen. However, due to low sperm density and irregular sperm orientation in these structures, I could not confirm the earlier workers' observations that these are the major sperm storage sites.

In this study the use of a radioactive label to identify the sperm of different inseminations proved to be only partially satisfactory. In autoradiography, the radioactive material is identified by correlating an exposed region in the emulsion with some structure immediately beneath the exposed region. If an isotope with a low beta particle energy, such as tritium, is used, a number of problems are encountered. The most significant is self-absorption by the tissue section. As section thickness increases, the efficiency of recording the radioisotope in the emulsion drops rapidly (Falk and King, 1963). In this study I had to compromise on section thickness to obtain a good set of serial sections, and as a consequence, I sacrificed efficiency. This is undoubtedly the reason why radioactivity could be detected only when spermatozoa were near the surface of the section. In addition to self-absorption, exposure to background radiation and contamination of sections, particularly by spreading the contents of ruptured cells across the sections, must be coped with in autoradiography. Ideally, the advantage of low energy radioisotopes is high resolution. However, in my
study, this advantage was negated by thick sections and continuous tearing of tissues during sectioning.

Since radioactivity was present in tubule sperm clusters when the second insemination contained labelled sperm, but was not present in sperm clusters when the first insemination contained labelled sperm, the sperm in the tubules of the treatment females must have come mostly from the second insemination. How these sperm come to predominate in the tubules is a matter of speculation.

Since sperm are found in tubules associated with all stages of oocytes, it is possible that sperm persist in a tubule until the oocyte matures. If this occurs, a second insemination can only dominate in these tubules by displacing the sperm already present. Alternatively, if the tubules are free of sperm at the time of the second insemination, sperm from this new insemination can take up the available space.

It is not known how long sperm persist in the tubules. In control females killed 20 days after insemination, sperm still persisted in the tubules. These females contained well developed embryos and therefore the oocytes associated with the tubules probably were not mature. However, although sperm may persist in the tubules for up to 20 days, I am not certain how many tubules in treatment females contained sperm from the first insemination when the females were re-inseminated. In these treatment females, not all tubules contained sperm. Consequently I cannot tell if sperm from re-inseminations displaced sperm already present.
or if sperm were stored in unoccupied tubules.

Regardless of whether or not sperm from the second insemination gain access to tubules by displacing pre-existing sperm, the observation that sperm from the second insemination dominate before eggs are ready to be fertilized explains the results of the previous two sections. When an insemination occurs during gestation, the sperm gain access to the tubules and persist in them until the associated oocytes are ready for fertilization. Since eggs are fertilized a few days after parturition (Rosenthal, 1954; Thibault and Schultz, 1978; Turner, 1937), sperm from re-inseminations immediately following parturition can also gain access to the tubules before mature eggs are fertilized, and thus may dominate. These sperm have precedence. If fertilization of a new brood of eggs begins before re-insemination, the re-insemination will not contribute as heavily to the next brood. Rosenthal (1952) has shown that the success of re-inseminations decreases as the time between parturition and re-insemination increases. Indeed, if sperm precedence is achieved in such a manner, the number sperm in a re-insemination will only have an affect if there are not enough sperm to gain access to all tubules associated with maturing oocytes.

It is unlikely that sperm are only stored in tubules associated with oocytes. Kadow (1954) and Jalabert and Billard (1969) identified diverticulae in the antero-dorsal portion of the ovary that can contain large numbers of sperm. The association between the spermatozoa and the
plasma membrane of the lumenal epithelial cells in this region indicates these are a site of long-term storage. In addition to this anatomical information, many commonly recognized reproductive phenomena in guppies suggest that sperm must persist in a general storage area. If sperm were stored solely in the tubules, the eggs of several broods would have to be present in the ovary when an insemination occurred. We know that up to eight broods can be fertilized from a single insemination (Winge, 1937). However, I have found only three groups of oocytes (representing different stages of development) in the ovary. Turner (1937) and Stolk (1951) also reported only three stages and this makes it unlikely that the eggs of eight broods would be developed enough to possess tubules. Also, in studies examining intraspecific sperm competition, the first insemination is, occasionally, re-instated after an initial dominance by sperm from the second insemination (Hildemann and Wagner, 1954). Again, if the tubules were the sole sperm storage sites, this change in sperm dominance could be accounted for only if sperm from re-inseminations were selective against tubules associated with the least mature oocytes. This does not appear to be the case (Winge, 1922; pers obs.).

The most tenable model at this time is that a more general sperm storage receptacle exists in addition to the tubules. At copulation, a portion of the sperm move to the tubules and a portion move to a receptacle in the antero-dorsal portion of the ovary. Sperm in the tubules fertilize oocytes as they mature. After parturition, another
copulation again sends sperm to both regions of the ovary. Those going to the tubules gain access before the oocytes mature, and as the oocytes mature, these sperm fertilize them before sperm from the previous insemination can gain access to the oocytes. Those sperm from the re-insemination which enter the sperm storage receptacle are stored in a position that gives them a competitive edge over already existing sperm, and thus give them precedence if a new insemination does not occur. As the sperm from the most recent insemination are used up, sperm from previous inseminations can again increase their success in fertilizing eggs. An examination of how sperm from different inseminations are distributed in the antero-dorsal ovarian storage site would give some insight into this problem.
General Discussion

A wide variety of female sperm storage systems occur in fishes. Although internal fertilization is typical of several families, sperm storage does not follow a consistent taxonomic pattern. Sperm storage occurs in sharks, surfperches (Embiotocidae), and poeciliids. Other groups probably also store sperm, but to date, there has been no attempt to survey the phenomenon in all fishes. Internal fertilization (a pre-requisite for sperm storage) is reported in some genera of cottids (i.e. *Clinocottus*, *Oligocottus*, and *Orthonopias* - Bolin, 1941; Morris, 1952, 1956). Morris (1952) reported a case of possible sperm storage in *Clinocottus recalvus*. In *Sebastes*, internal fertilization and ovoviviparity also occurs, but too little is known about reproduction in this group to comment on the possibility of sperm storage (Breder and Rosen, 1966).

All three fish groups that are known to store sperm possess strikingly different modes of reproduction. There are differences in their female reproductive systems, and consequently in their modes of sperm storage. In addition, the reproductive ecology of each group is sufficiently different to suggest that sperm storage may serve different functions in each group.

The shark reproductive system has a typical vertebrate organization. As in all other vertebrates that store sperm, sperm are stored, and fertilization takes place, in the
reproductive tract. Metten (1941) reports sperm in the tubules of the oviducal gland in *Scylliorhinus canicula*. In the blue shark, *Prionace glauca*, spermatozoa occur in the tubules in the posterior shell-secreting portion of the oviducal gland (Pratt, 1979). Since a functional shell is not produced in this species, Pratt suggests the shell-secreting tubules have taken on a new function – sperm storage. The ultrastructural details of sperm storage in sharks are unknown.

The duration of sperm retention in females is unknown in most species, but in female blue sharks, sperm may persist for one to two years (Pratt, 1979). In this species, five year olds mate in late spring, but fertilization does not occur until the following spring. Gestation takes 9-12 months and another mating is probably required to replenish the sperm supply (Pratt, 1979). Since fertilization is delayed for a year, sperm storage is an essential part of blue shark reproduction. Unfortunately, the functional significance of delayed fertilization in this species is not apparent.

Embiotocids and poeciliids, although phylogenetically unrelated, have similar modes of reproduction. Their reproductive systems are typical of teleosts. Females have a single, hollow ovary (fused paired ovaries) which connects to the exterior via a short duct. Both groups are live-bearers and store sperm in tubules or diverticulae in the ovary. Their reproductive cycles, however, differ drastically and as a consequence, sperm storage appears to
serve different roles in the two families.

Embiotocids, or surf-perches, are inshore, marine percoids. They are endemic to the north Pacific Ocean. Eigenmann (1892) was the first to note that male and female reproductive cycles are six months out of phase. He studied Cymatogaster aggregata and discovered that sperm were stored for prolonged periods. Apparently, females are inseminated in the summer and fertilization occurs in the winter when eggs are mature. Gestation takes approximately six months (Turner, 1952; Wiebe, 1968). Sperm are stored in pockets in the lumenal epithelium and are intimately associated with the epithelial cells (Gardiner, 1976). Ultrastructural investigations reveal that sperm are embedded in invaginations of the cell plasma membrane, much as occurs in other sperm storing vertebrates (Cuellar, 1966; Hoffmann and Wimsatt, 1972; Wimsatt et al, 1966).

As in sharks, sperm storage apparently plays an essential part of embiotocid reproduction. Given the long gestation period (Turner, 1952; Wiebe, 1968), delayed fertilization is likely a strategy to ensure that critical stages in embryo development do not occur during the winter and that young are born in late spring or early summer when food may be more available. It is not known if sperm persist in the ovary beyond fertilization.

In contrast to embiotocids, the female reproductive cycle in poeciliids is short, ranging from about 22 days to 40 days (Colson, 1969; Turner, 1937; van Oordt, 1928; Winge, 1937). For short periods in each cycle, females are
receptive and fertilization generally occurs a few days after insemination (Dildine, 1931; Rosenthal, 1952; Turner, 1937). Sperm can remain viable in the ovary for several months and fertilize each new batch of eggs as they mature (van Oordt, 1928; Winge, 1937). This situation is unique in the vertebrates. Females generally mate with more than one male (Borowsky and Kallman, 1976; Haskins et al., 1961) even though sperm from each insemination persists in the ovary. Consequently the role of sperm storage in poeciliid reproduction is not clear.

The fact that female poeciliids are able to maintain reproductive output when sperm availability is low suggests that sperm storage may be an adaptation to ecological factors that affect the availability of males (e.g. selective mortality on males, drought and flooding which might leave females isolated from males). Sperm storage would allow females, in the absence of males, to colonize new habitats or recolonize areas subject to ecological catastrophe. However, evidence to suggest that female poeciliids commonly face shortages in sperm supply is circumstantial. Poeciliids live in a variety of habitats ranging from fast-moving, deep streams to shallow, lowland streams and ponds. In fast-moving streams, females may be washed downstream, but there is no evidence that females swept to another area can establish a new population or that this is a frequent occurrence. In lowland ponds and streams, floods may also sweep females into new areas, but again the frequency of this occurring is unknown. Flooding
may allow colonization of new areas or recolonization of previously populated areas. However, it seems unlikely that only females move into these newly flooded regions. In Trinidad, ponds containing guppies dry up in the dry season and are flooded again in the wet season (Liley, pers. comm.). Liley observed that males and females recolonized these flooded areas together.

Sex ratios skewed in favour of females have been reported in populations of some poeciliids (Krumholtz, 1963; Seghers, 1973). If this skewing of sex ratios is common in poeciliid populations and creates a sperm supply shortage, sperm storage may be an adaptation to low male availability. Unfortunately, it is not known how skewed the sex ratio must be to produce a sperm supply shortage. Since females are receptive for only part of their reproductive cycle, the effective sex ratio at any given moment is probably different from the observed sex ratio. Thus, for a shortage in sperm supply to exist, the proportion of males in populations may have to be much lower than normally observed.

Since poeciliid females are capable of storing sperm for relatively long periods, it is curious that females remain susceptible to further inseminations. A single mating provides enough sperm for more than one brood and only when the sperm supply in the ovary dwindles do females require another insemination. The fact that females of some poeciliids ( Xiphophorus maculatus and Poecilia reticulata ) are inseminated by more than one male suggests that multiple
insemination combined with sperm storage has some adaptive value. Multiple insemination in poeciliids means that females can accumulate sperm in their ovaries. Billard (1966) and Zander (1962 - cited by Borowsky and Kallman, 1976) reported that in *Poecilia reticulata* and *Xiphophorus* brood size increases as the number of spermatophores (and hence the amount of sperm) in inseminations increases. These results suggest that if the number of spermatophores transferred during insemination is small, storing sperm from these inseminations may result in increased brood sizes.

Unfortunately, the number of spermatophores transferred during insemination is unknown. Billard's (1966) data indicated that in *Poecilia reticulata*, broods resulting from artificial inseminations with 100 spermatophores were slightly larger than broods produced after natural inseminations. This would suggest that if sperm from artificial inseminations are as effective as sperm in natural inseminations, less than 100 spermatophores are transferred in natural inseminations. If sperm from artificial inseminations are less effective than sperm in natural inseminations, natural inseminations may contain considerably fewer than 100 spermatophores. Since the mean brood size obtained from all inseminations by Billard (1966) was much less than brood sizes reported by other workers (Rosenthal, 1952; Thibault and Schultz, 1978; pers. obs.), possibly the amount of sperm in one of Billard's inseminations was not enough to produce the maximum brood size. Results from guppy mate selection experiments by
Gandolfi (1971) provide further evidence that the number of sperm in a single insemination is insufficient to produce the largest brood. Females inseminated by two males produced broods almost twice as large as broods produced by females inseminated by a single male. Thus, if accumulation of sperm in the ovary has the same effect as increasing the number of sperm in inseminations, sperm storage may be a means for females to maximize reproductive output.

Since female guppies expend more in reproduction than males and hence would probably lose the most from mistakes in mate selection, females are expected to be more selective than males when choosing a mate. Indeed, female guppies do appear to be selective in their choice of males to mate with (Gandolfi, 1971; Parr, 1976, 1977). Although female guppies may store the sperm from one male, if a more fit male comes along, and the female can perceive male fitness, she would benefit from mating with the male and thus, she would be expected to mate with the superior male. Since sperm from the most recent insemination fertilize most or all of the following broods until it is gradually replaced by sperm stored from earlier inseminations, the phenomenon of sperm precedence may maximize the benefit of matings with more fit males.

The anatomical model that I proposed in Section IV to explain sperm precedence does not necessarily conflict with the proposal that accumulation of sperm in the ovary may increase brood size. If inseminations are so small that sperm does not reach all tubules associated with mature
oocytes, sperm from earlier inseminations, stored in the antero-dorsal diverticulae of the ovarian lumen, may move into the remaining tubules. This means that inseminations containing a very small number of spermatophores may not show sperm precedence. The number of spermatophores in my artificial inseminations were probably large enough for sperm to reach most tubules associated with mature oocytes and hence in my experiments, sperm from re-inseminations fertilized most of the mature oocytes.

One of the basic components of sperm storage in vertebrates is maintenance of spermatozoa. Presumably sperm remain inactive during storage (Wimsatt et al., 1966) and then are reactivated when mature oocytes become available. The physiology of sperm storage maintenance during storage has received some attention (Gardiner, 1976; Hoffmann and Wimsatt, 1972; Racey, 1975). Since in most vertebrates that store sperm, sperm are closely associated with the plasma membrane of the epithelial cells of storage sites, it is postulated that these epithelial cells provide nutrition for sperm. In bats, Racey (1975) found an abundance of glycogen, enzymes associated with conversion of glycogen to monosaccharides, and fructose present in the uterine fluid (uterus is sperm storage site in bats). Since mammalian sperm are capable of aerobic respiration and fructolysis, possibly the substances in the uterine fluid of bats sustain the stored sperm. The production of potential sperm metabolites by cells of sperm storage sites of sperm storing teleosts has not been investigated. However, sperm from
Cymatogaster aggregata and Poecilia reticulata are capable of metabolizing extracellular glucose (in fish with external fertilization, sperm must rely on endogenous glucose) and Gardiner (1976) postulated that sperm from these species obtain nutrition from ovarian lumenal epithelial cells at storage sites.

In contrast, virtually nothing is known of the mechanisms controlling sperm deactivation and reactivation or release from storage. Dent (1970) noted that a myoepithelial layer around the spermatheca of the red-spotted newt and suggested that contraction of this layer expels sperm into the reproductive tract. Fox (1956) postulated that in garter snakes, eggs moving down the reproductive tract squeeze sperm from the storage tubules in the reproductive tract. More recently, however, Hoffmann and Wimsatt (1972) found that the epithelial cells of sperm storage sites in garter snakes become vacuolated and suggested that this may be related to release of stored sperm. In Poecilia reticulata, cells of the antero-dorsal diverticulae also appear to become vacuolated and Jalabert and Billard (1969) suggest that this vacuolation may be related to sperm release from storage sites. Unfortunately, these are merely casual observations from anatomical studies and no attempt has been made to test these speculations.

In Poecilia reticulata, fertilization of a new batch of eggs by stored sperm occurs a few days after parturition (Rosenthal, 1952; Thibault and Schultz, 1978; Turner, 1937). This time lapse between parturition of one brood and
fertilization of the next brood may result from a lapse between parturition and sperm release or a lapse between parturition and egg maturation. However, Rosenthal's (1952) study suggests that release of sperm from storage sites is delayed. Sperm from new inseminations immediately after parturition fertilize most of the next batch of eggs. If stored sperm were released immediately after parturition, it is unlikely that sperm from the new insemination would dominate fertilization.

Factors controlling release of stored sperm from storage sites, vitellogenesis, and parturition may be linked in some way to produce a coordinated system. Secretion of gonadotropic and ovarian steroids are associated with vitellogenesis, increased sexual behaviour (Liley, 1972), and perhaps, secretion of a sexual pheromone (Crow and Liley, 1979) in the guppy, possibly these hormones also control, directly, or indirectly, the release of stored sperm from storage sites. However, at present there is no direct evidence available.

In conclusion, although the functional significance of sperm storage in guppy females is not clear, the organization of sperm storage gives guppy females considerable flexibility in reproduction. If sperm availability is low at times, females can maintain reproductive output. In addition, accumulation of sperm from more than one insemination may enhance brood sizes, but at the same time, sperm from new inseminations can dominate over stored sperm during fertilization. Thus, although
sperm storage may maximize reproductive output, females can benefit immediately from matings with superior males. Anatomically, the guppy ovary appears to be structured so that stored sperm can fertilize several broods, but also, so that fresh sperm can have precedence over stored sperm. Unfortunately, some of the details of sperm activity in the ovary are lacking. The time of reactivation of stored sperm relative to parturition and whether fresh sperm dominate in the oocyte tubules by entering unoccupied tubules or by displacing older sperm that are present in tubules are factors affecting sperm precedence. Possibly investigations of these factors will clarify the actual mechanism of sperm precedence.
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