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Investigating a Method to Measure Sperm Transfer in Chelidonura sandrana (Opisthobranchia: Cephalaspidea)

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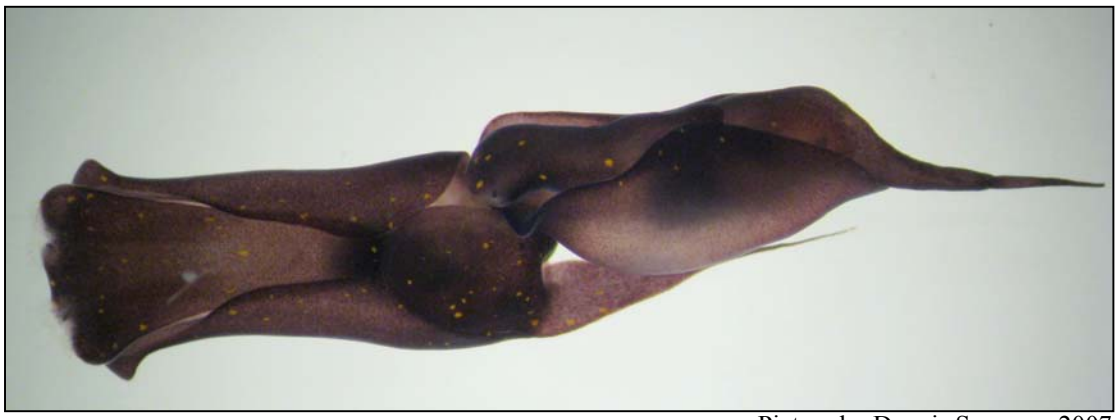
Investigating a method to measure sperm transfer in *Chelidonura sandrana*

(Opisthobranchia: Cephalaspidea)



Picture by Dennis Sprenger 2007

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Abstract

This paper investigates possible methods for measuring sperm transfer in the internal fertilizing, simultaneous hermaphrodite *Chelidonura sandrana* (Opisthobranchia: Cephalaspidea). Comparing sperm amount transferred in copulations has significance for testing the assumption that sperm transfer is linearly correlated with copulation duration as well as providing a tool for future studies.

Various methods of preparing, treating, and viewing sperm samples were attempted. Two unsuccessful pilot studies were conducted to test free sperm counts and measuring sperm pellet surface area. Future research should focus on optimizing centrifugation method for surface area measurements of sperm clusters and resuspending sperm clusters to enable sperm counting. In conclusion, this study provides a background for future work measuring sperm transfer in *C. sandrana*.

Keywords: *Chelidonura sandrana* – Opisthobranch – sperm transfer – methodology

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Acknowledgements

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Figures

Tables

1. Introduction

1.1. Background

In simultaneous hermaphrodites (both sexual functions expressed concurrently) reproductive success is a function of energy allocation to each sex role (producing eggs or sperm) (Connell & Gillandres 2007). Optimal function in both sex roles will inevitably create conflicts in both resource allocation and mating behaviour within a single organism. Conflicting gender roles are of particular interest in the field of evolutaionary biology, particularly in regards to sexual selection (Anthes 2005). Sperm trading (conditional and reciprocal sperm exchange between internally fertilizing hermaphroditic mates) has been postulated as a mechanism to resolve conflicts in simultaneous hermaphrodites by equalizing the net fitness costs and benefits for each mate (Leonard and Lukowiak 1984). Sperm trading is established in several organisms (see Michiels et al. 2003; Vreys and Michiels 1998; Anthes 2006). In the sea slug *Chelidonura sandrana* (Opisthobranchia: Cephalaspidea), an internal fertilizer which transfers free sperm (not spermatophores), the presence or absence of sperm transferred during copulation does not affect reciprocity (Anthes 2006).

1.2 Recent research and sperm transfer in *C. sandrana*

Recent studies on *C. sandrana* focus on the effects of multiple mates on offspring viability. Sperm diversity increases both egg capsule size and veliger (free swimming molluscan larvae) size (Sprenger 2007). Sprenger (2007) compared offspring between individuals mated once with the same individual, multiple matings with the same individual, or the same number of matings with a different individual each time. This experiment found that offspring size increased with multiple mates rather than repeated matings with the same individual, indicating that sperm diversity, not amount, accounts for difference in offspring size. This experiment assumed that

repeated matings transferred roughly the same amount of sperm, the only difference being whether it was from one or many individuals. This assumption was rationalized by the link between penis intromission duration to insemination duration and corresponding ejaculate size (Sprenger 2007). Also, copulation times remained constant for individuals over repeated matings (up to 8 times in 9 hours) (Sprenger 2007). This indicates that total sperm transferred remained constant for an individual.

Penis intromission is correlated linearly with insemination duration (R^2=0.82) (Anthes 2006 p. 61). Observations of regularly spaced transfer of equally sized sperm strains during insemination indicate a positive linear correlation (Anthes 2006 p. 64)

Anthes, 2006, established that male acting individuals adjust copulatory behaviour to altered mating status of their mate (when acting females had been isolated, their mates increased intromission time) but not to concurrent mating behaviour.

Observances indicate that sperm transfer is constant over time in *C. sandrana* and that individuals vary the amount of sperm transferred only by adjusting copulation duration. The assumption that copulation time reflects relative amount of sperm transferred has been used to interpret previous studies on reproductive behaviour in *C. sandrana* (i.e. individuals that copulate longer transfer more sperm). However, how insemination duration relates to sperm amount has not been measured (Anthes 2006).

1.3 Measuring sperm transfer in simultaneous hermaphrodites

Previous studies have dealt with individuals that transfer encapsulated sperm and/or externally transfer sperm (see Locher & Barr 1997; Rogers & Chase 2001; Longley & Longley 1984).

1.4 Study Goals

This study will investigate methods of evaluating the amount of sperm transferred over time for *C. sandrana.*

Being able to measure the number of sperm transferred between individuals is of interest in resolving questions about reproductive strategies in simultaneous hermaphrodites. Of particular interest is a method to establish the relationship between copulation duration and amount of sperm transferred. If the amount of sperm transferred differs according to copulation duration in a linear fashion, it indicates constant transfer rates over time per individual. If such is the case, insemination duration clearly reflects the amount of sperm transferred. Since there is such a close relationship between intromission duration and insemination, intromission can reliably used as an indicator of the amount of sperm transferred.

To general knowledge, measuring sperm transfer has never been done before in Cephalaspideans. Working with an internal fertilizer which transfers free sperm in short bursts poses several challenges including:

1) Obtaining the sperm without interrupting normal mating behaviour.

2) Free sperm is transferred rather than encapsulated packets, which complicates removal of all sperm during a mating.

The anatomy of *C. sandrana* yields several opportunities for studying sperm transfer. Spatial separation of copulatory organs enables easy discernment of the gender role in which the animal is currently involved. The sperm groove and translucent penis enable visual observation of sperm transfer during normal copulation. If the sperm groove is interrupted, sperm transfer is suspended, and sperm is released into the water. However, cauterization of the sperm groove so that the individual is unable to donate sperm produces no difference in the copulatory behaviour of mating pairs (Anthes 2006).

2. Methodology

2.1 Study Organism

Chelidonura sandrana was used as a model organism in this study. *C. sandrana* is a small (less than 20mm) opisthobranch found in different colour morphs from the Red Sea to the Maldives and Western Australia (Debelius 1996). *C. sandrana* is found in relative abundance around Lizard Island ($14^{o}40$'S $145^{o}26$'E), is easily maintained in the laboratory, has diurnal mating patterns with copulations lasting from a few minutes to an hour, and has been established as a model species (Anthes 2006). *C. sandrana* is an out-crossing (non-self fertilizing), internally fertilizing simultaneous hermaphrodite with the ability to store donated sperm. Their mating clearly illustrates reciprocity; after some initial intertwining the animals will come to a mutual decision of gender roles in which one acts as the male first; then roles are switched. After an intromission (the period from entrance of the penis into the female genital opening until male retraction) of 10-20min the animals switch sex roles and repeat the process (Anthes 2006).

The general body structure of *C. sandrana* is as follows (Figure 1). The body consists of two major portions- a cephalic and posterior shield. Two tails are present, one significantly larger than the other. A parapod along each side meets over the top. Reproductive organs are located under the right parapod, with separation of the male and female copulatory organs. The male copulatory organ ("penis") is located on the head, and inverted until copulation. A ciliated external sperm groove runs along the right parapod, connecting the penis to the gonopore (female genital opening). Sperm entering the gonopore travel into either the bursa copulatrix (in which sperm are phagocytised) or a seminal receptacle in which sperm from multiple mates are stored until the time of fertilization (Anthes 2006).

Figure 1. *C. sandrana* ready for cauterization, with parapoda unfolded to expose the cephalic and posterior shields, and showing the tails, external sperm groove, and sensory bristles along the head.

2.2 Specimen Procurement

This study was conducted from November 03-30, 2007 at Lizard Island Research Station on the Great Barrier Reef. Specimens were collected from sand at depths of 0.5-1.5 m at Mangrove Beach, Lizard Island under Great Barrier Reef Marine and Parks Authority (GBRMPA) permit number GO5/15308.1. Specimens were collected by snorkelling with use of a 1-mL disposable pipette with tip cut off. Individuals were returned to approximately the collection location after laboratory use. Specimens were kept in 75mL vials and water was exchanged daily. Specimens were kept at 26^{o}C and fed periodically with the flatworm *Wulguru cuspidate*, their natural food source (Sprenger 2007).

2.3 Cauterization

Cauterizations followed a modification of Anthes, 2006. Specimens were anesthetized in concentrated $MgCl^2$ diluted with seawater for 15min. Water was removed and the right parapod brushed aside to expose the external sperm groove (visible as a white line). A thin need was heated in a Bunsen burner and applied to the

sperm groove at the junction of the cephalic and posterior shields. Specimens recovered approximately 24hr.

2.4 Sperm Samples

Cauterized individuals were mated in 0.5µm filtered seawater (purified sH_2O. Sperm (visible as a white string and/or cloud emerging from the posterior or right parapod of the animal) was collected with use of 15µL or 200µL pipettes. Matings occurred within 48hr of cauterization, as individuals healed and were able to transfer sperm again within 2-3days. The times copulations started and ended were recorded and averaged.

2.5 Concentration

Centrifugation and evaporation were used to concentrate sperm samples. Samples were centrifuged in 1.5mL Eppendorf pipettes with a microcentrifuge at the speeds and rpm listed in Table 1. Samples were evaporated in a laboratory oven at 60^{o}C. If necessary, samples were rehydrated with purified seawater and methylene blue dye. Water from mating was filtered through 0.22µL pore-size cellulose-nitrate filter paper.

2.6 Homogenization

A procedure modified from Longley (1984), Locher and Baur (1997), and Rogers (2001) was used to homogenize a sample which had been stored at 4^{o}C for over 24hr. The sample was homogenized with a 200µL Eppendorf pipette tip cut to an inner diameter of 2mm for 10min; the Eppendorf pipette was set on 100µL and the sample was homogenized for 10min with a tip cut to 1mm; the sample was homogenized for 10min with a tip of inner diameter 0.5mm. All homogenizations were done at a rate of 1/s. The sample was checked for sperm before each homogenization.

2.7 Preservation

Samples were monitored over time for changes in amount of free sperm numbers. Refrigeration chilled samples to 4^{o}C. Ethanol and methanol were added to samples and the number of sperm compared over time.

2.8 Viewing

Slides with approximately 5µL of sample were viewed with a Leitz Dialux22 light microscope at 1000x magnification. Dye was used to stain sperm heads.

2.9 Haemocytometer sperm counts

A 0.100mm-hemocytometer was used to view and count sperm at 250x magnification. A haemocytometer has 2 counting chambers divided into grids of 9 squares. The central square, or central counting chamber, is divided into 16 large squares, each of which is divided into 16 small squares. Each chamber holds 15µL of fluid, and each of the nine squares on the grid has an area of 1mm^2, and the coverglass is 0.1mm above the chamber floor. A known volume of 0.1mm^3 (=0.1µL) is over the central counting chamber along with a dilution factor and the original sample volume, allows calculation of the number of sperm present in the original sample.

2.10 Hematocrit

Eight plastic clad micro hematocrit tubes (Ammonium heparin) tubes were filled with a B sample, sealed with clay, and centrifuged in a Micro Hematocrit Centrifuge in 3 increments of 5min.

2.11 Sperm counting pilot study

Matings between two cauterized individuals were observed on a dissection microscope at 12x magnification. Body wet-weight (mg) was measured using an electronic balance and mating pairs were sized matched between individuals (±25%, size range 6.6-34.1mg; mean 15.4mg). Visible sperm was collected with a 200µL

Eppendorf Pipette. Mating individuals were randomly assigned to one of two groups. Group A measured the sperm transferred after the first 5min of mating. Group B individuals were allowed to complete the entire copulation. After the first mating was complete (signalled by penis disengagement) the pair was pipetted out of the watch glass into fresh water. Individuals were identified by characteristic features, and repeat matings were stopped by pushing the individuals apart with a small paintbrush. Mating start and stop times were recorded. Samples were centrifuged at 1000rpm for 1min, and the average number of sperm on the counting grid recorded. Total sample volume was estimated by pipetting the residual volume out with an Eppendorf pipette set to known volumes and adding the volume used on the haemocytometer.

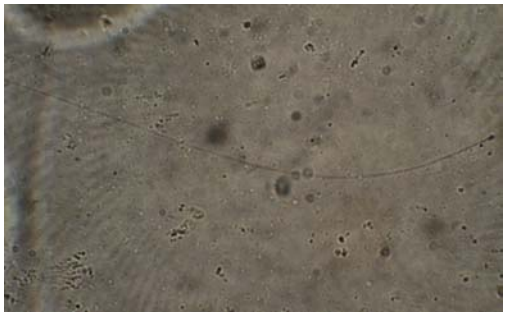
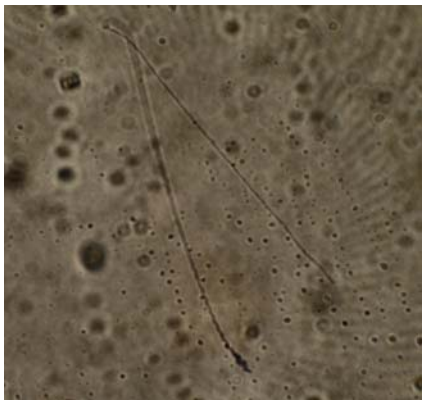
2.12 Surface area pilot study

Mating set up and sperm collection were the same as in the previous pilot study (size range 4-25mg; mean 12.3mg). Observations and experiments took place within 48 hours from collection. Samples were centrifuged for 10min at 8000rpm and the bottom150µL placed on a slide with large coverslip raised to standard height. Sides of the vials were scrapped with the pipette tip to remove any sperm stuck to the edge of the vial. At 40x magnification, 4 visual transects were done along the slide and clusters were photographed with a 3.3 or 5.0 MegaPixel Olympus digital camera. Pictures were analysed for pellet surface area (mm^2; not corrected for sperm density) using the ImageJ image analysis system. Because sperm often occurred on mucus strands, surface area calculations were inclusive of mucus in pellets.

3. Results

3.1 Microscope images

Sperm were nonmotile and extremely elongate and thin with pointed, corkscrew heads (Figure 2).

a.  b. 

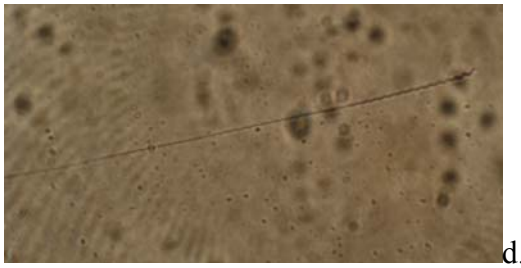
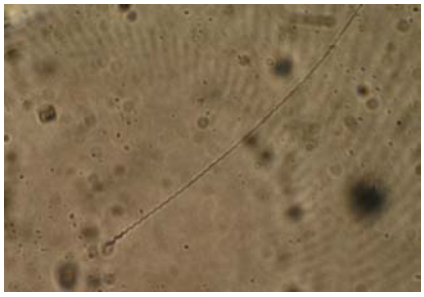
c.  d. 

Figure 2. Sperm at 1000x magnification on a light microscope showing general appearance (a,b) and corkscrew shaped head (c,d) (5Mp 50/50 Olympus).

A 200µL-Eppendorf pipette was more effective at collecting sperm samples. verage copulation duration for full matings was 11.34min (range 4-30min). Sperm was visible 1-3 minutes after intromission began. In some cases, sperm stopped being visible a few minutes before penis retraction while in others it was released until separation.

3.2 Concentration

Fresh samples were either too dilute to achieve significant numbers on the haemocytometer or sperm clustering occurred, sometimes obscuring the counting grid. Lower counts of free sperm were often associated with clusters. Centrifugation was done at varying speeds (1000-10000rpm) and durations (30s- 30min). Centrifugation speeds, times, and qualitative results are summarized in Table 1.

Centrifuge (rpm)	Time spun (min)	Evaporation (min)	Result
<2000	0.5	Fully evaporated (254)	No sperm
<2000	0.5	" (342)	No sperm
<3000	0.5		Few sperm
<3000	0.5	Evaporated to one drop (132)	Increased sperm numbers from previous sample
1000	1		Free sperm condensed to bottom; clustering
1000	2		Small sperm clusters
1000	2.5		"
1000	3		Clusters and distinct pellet discernable
1000	4		Pellet but many free sperm
1000	5		Pellet but free sperm
1000	6		"
1000	7		"
1000	10		Pellet and few to no free sperm; larger samples contain small clusters
1000	12		All samples pellet
1000	15		nothing
10000	10		No sperm*
10000	15		"
10000	15	Fully evaporated (254)	"
10000;5000	10;5		"
10000;5000;2000	10;5;10		"
5000;10000	34;5		"

Table 1. Centrifugation speeds and times attempted in this experiment, along with subsequent drying of samples. < [rpm] indicates that the sample was placed in the centrifuge and the speed was turned up to that rpm and then back down immediately so that the sample was spun for approximately 30s.*Samples were checked for the presence of free sperm and not clusters.

Centrifuging condenses free sperm towards the bottom of the tube.

Centrifuged samples were checked for free sperm in the top half of the water. Free

sperm were still found after centrifuging for 4min at 1000rpm, but few (1-2) to no sperm were found in the sample after 10min at 1000rpm.
Centrifuging at 1000rpm was effective in concentrating/pelleting sperm as well as non-destructive to sperm samples.

Results were extremely variable between samples. Centrifuging for 12min at 1000rpm for samples on one day created a large pellet visible by eye. The same processing produced small clusters of sperm in samples when done the next day.

Samples in this study took 132-1500min, depending on volume, to evaporate in an oven at 60^{o}C. Attempted rehydration of sperm samples in which water was completely evaporated was unsuccessful.

Filter paper (pore size 0.22μm) was too opaque to view filtered samples on a light microscope.

3.3 Homogenization

The sperm count dropped immediately after the first homogenization (from 17 to 0). All attempts to resuspend pellets or free sperm from clusters resulted in decreased sperm counts.

3.4 Preservation

Throughout the day, no difference in sperm counts could be established. Sperm counts decreased by the next day (from 17-9 sperm per central counting area of the haemocytometer grid). Sperm numbers decreased even more by the second day.

Refrigeration overnight showed a slight decrease in sperm number that fell within the range of variation for successive sperm counts of the same sample.
In samples compared after sitting for 8 hours in the lab (26^{o}C) and refrigerated samples (4^{o}C) no apparent difference was observed.

No difference was observed between samples with or without ethanol for samples left to sti for 1-2 days. Samples read after 2 days showed no sperm whether or not Ethanol had been added. Methanol likewise produced no change in sperm samples.

3.5 Viewing

A normal sized coverslip was too little volume (5μL) to accurately assess sperm concentration. For viewing pellets, a larger volume (150μL) produces the best results. Sperm were easily recognizable at 400x magnification on slides and 250x on the haemocytometer. Dye was unnecessary

3.6 Haemocytometer

Haemocytometer counts works for counting free sperm but sperm clusters complicate sperm counts.

3.7 Hematocrit

Pellets could be observed on the clay but could not be measured.

3.8 Sperm counting pilot study

The results of the sperm counting pilot study are in Table 2.

Group	Body Weight (mg)	Treatment	Copulation Duration (min)	Count 1	Count 2	Count 3	Count 4	Average	Total Sample Volume (µL)
A	9.8	1 drop MeOH	5	55	34	4	5	24.50	275
A	11.5	10µL EtOH	5	10	4			7.00	140
A	14.3	Centrifuged 1min @1000rpm	5	21	20	8	7	14.00	280
A	16.2	"	5	28.00	13			20.50	465
A	19	processed immediately	5	3	2	4	2	2.75	130
A	18.8	"	5	1	1	2		1.33	175
A	19	"	5	56	16	7	12	22.75	185
A	12.9	"	5	36	16	25	30	26.75	190
A	7.5	"	5	320	0	4	1	81.25	275
B	20.5	Centrifuged 1min @1000rpm	10	400.00	32			216.00	445
B	15.6	"	9	960	70	23	24	269.25	225
B	9.2	processed immediately	12	1	1000	0	2	250.75	950
								22.31	

clusters affected counting

Table 2. Results of initial pilot study. Counts are for numbers of sperm found on haemocytometer central counting area. Group A average: 22.31sperm per central counting area, range: 1.33-81.25. Group B average: 245.33sperm per central counting area, range 216.00-250.75. Clusters are explained in Section 3.7.

Groups were not represented equally between the groups because individuals were assigned a random group pre-copulation. Sometimes only one of two potential matings per pair happened if slugs missed each other when changing roles, an individual was not cauterized, or the mating happened in such a position as to preclude sperm collection.

Group B samples were higher than Group A samples (Figure 3).

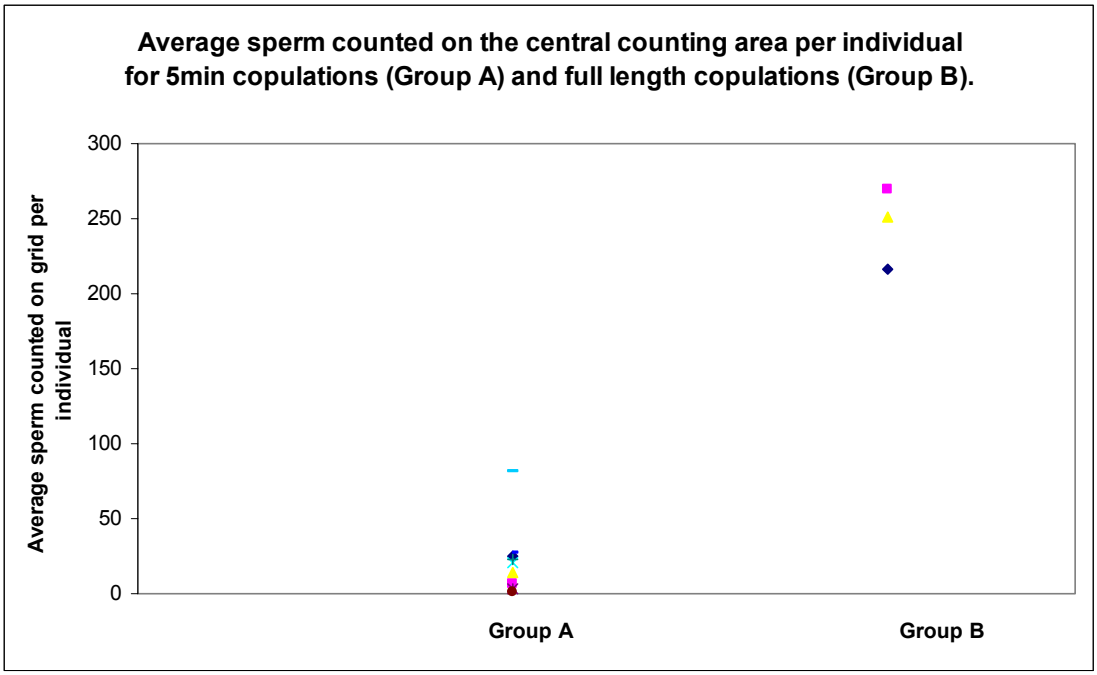


Figure 3. Average sperm counts per individual on the central counting chamber of the haemocytometer for individuals in Groups A (5min) or B (full length copulation).

Clusters were composed of aggregations of sperm with or without mucus strands. A cluster viewed at various magnifications is shown in Figure 4. Sperm density ranged from an indistinguishable mass of sperm to a loose aggregation. Sizes varied; covering the whole viewing field or more at 250x magnification on the light microscope (visible by eye both in the Eppendorf tube and on the haemocytometer grid) to small pellets of 5-10 sperm. Of 16 samples in the original pilot study, clusters were observed in 9 samples. Clusters on the haemocytometer (but not necessarily on the counting grid) were found in 5 of the 10 Group A samples. Four of six B samples read contained clusters on the haemocytometer (but not necessarily on the counting grid). Four samples had clusters directly over the grid so that they precluded counting. Since no method could universally be determined to count sperm in clusters, the pilot study was terminated. Because the pilot study was terminated, insufficient data was collected for analysis of sperm counts by body weight and specific copulation duration.

a.  b. 

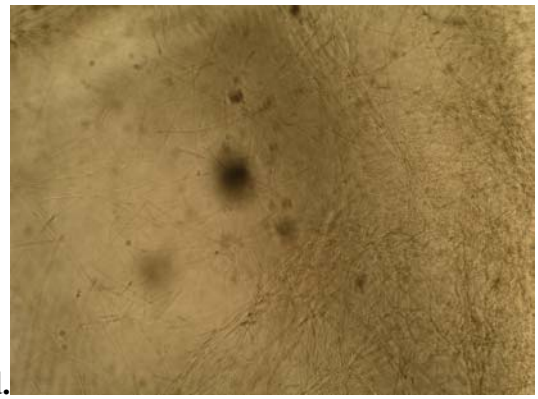
c.  d. 

Figure 4. Sperm cluster viewed at 40x (a), 100x (b), 250x(c), and 400x (d) (5Mp 50/50 Olympus).

3.9 Surface area pilot study

Pellets did not form as previously documented for centrifugation at 1000rpm for 12min. Instead, numerous smaller clusters occurred in the sample (Figure 5).

a.  b. 

Figure 5. Sperm clusters viewed during surface area counts (40x; 3.3Mp Olympus).

Samples were centrifuged again for 10min at 8000rpm with no change in sperm content. Samples were also spun for 10min at 1000rpm and 5min at 1000rpm with no

change in sperm content. Since no change occurred in samples with variations in centrifugation rpm and time, data was pooled to generate Figure 6.

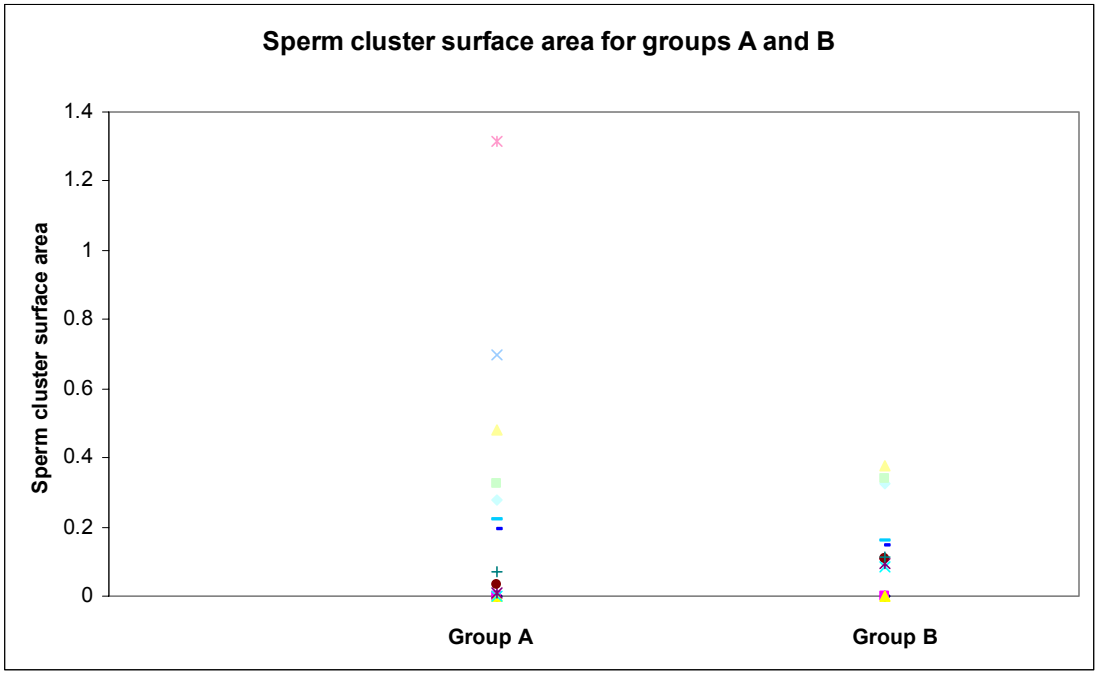


Figure 6: Sperm cluster surface area for groups A and B. Group A average: 0.2589, range 0-1.315. Group B average: 0.1457 range 0-0.377.

This data shows that B individuals transfer less sperm than A individuals on average.

4. Discussion

4.1 Viewed Sperm

Non-motile sperm are as expected for an out-crossing species. To avoid self-fertilization some species use a mechanism by which self- sperm is activated in the seminal receptacle of the female-acting mate (Anthes 2006).

4.2 Techniques for processing sperm

4.2.1 Centrifugation

Centrifugation provided the most practical method of concentrating sperm samples. However, this method proved unreliable universally. The effects of

centrifugation should be related directly to the number of sperm in the sample and are dependent on sample quality. Additionally, sample destruction is a possibility at higher speeds. Although samples usually become more concentrated with time and/or speed, sperm are so small that they would be in danger of fragmentation at high velocities.

4.2.2 Preservation

Although no difference in sperm samples could be empirically established over time, use of fresh samples (within 1-3 hours) is recommended. Use of fresh samples prevents a potential unknown in the sample results. This disagrees with previous sperm transfer studies by Longley and Longley (1984), who measured sperm transfer in the nudibranch *Aeolidia papillosa* (externally transfers sperm packets). Sperm were collected by pipette during copulation or extracted from an ampulla (sperm storage organ) and stored at 4^{o}C for 2 days, before being dissociated, diluted, and counted on a haemocytometer.

4.3 Sperm counting pilot study

The largest problem in the initial pilot study was the clustering of fresh sperm samples. Because clusters influenced counting by either preventing accurate sperm counts or reducing the number of free sperm in the water, sperm counts did not reflect a plausible relationship between sperm transfer amounts over time, showing an exponential rather than linear relationship between 5min and full length copulations. However, data was skewed by counts affected by clustering.

The strengths of this type of study are quantitative measurements of sperm amounts. Weaknesses are that no viable method for resuspending clusters has been found.

4.4 Surface area pilot study

Results show the exact opposite of the expected trend, in that full length copulations appeared to have pellets with smaller surface areas. The largest problem in the secondary pilot study was the irreproducibility of the centrifugation methods. Sperm did not form a large pellet but either formed several small pellets or the sample lacked pellets at all.

The strengths of this method are that with determination of a universally applicable centrifugation method, this would provide accurate and easy comparisons of the amount of sperm transferred. Centrifugation (~10min at 1000rpm) removes free sperm from the sample, indicating that all available sperm is viewed at once. The weaknesses of this method are that it does not account for sperm cluster density, pellets also contain mucus strands and other excreted substances, and it is not as specific as sperm counting.

Although this method mirrored the work of Michiels & Streng with *D. polycroa,* the planarian transfers sperm in densely packed clumps. Sperm transfer involves injection of seminal fluid into the bursa, later followed by the sperm clump. Michiels & Streng (1997) isolated these allosperm clumps from the female reproductive tract of previously mated individuals and took surface area measurements. Sperm clump density in *D. polycroa* was found to be consistently dense, while only a few samples were small loose aggregations of sperm. Attempting this method in *C. sandrana*, in which transferred sperm are free and clustering is a secondary affect, is more difficult as clusters are irregular in abundance, concentration, and density.

4.5 Error

Limitations on any method of measuring sperm in *C. sandrana* are posed by mating behaviour of the individuals, the effectiveness of the cauterization, and the quality of extraction. Excessive disturbance of the animal during pipetting could cause retraction or a pause in sperm transfer.

Potential causes of imprecision that were not investigated in this experiment include a possibility sperm sticking to the sides of the plastic Eppendorf tubes or within the Eppendorf pipette tip.

Mating behaviour limited sperm extractions in some cases. Mating rates varied with time of day (greater in morning than afternoon) and time of month (greatest around the full moon). Mating position was variable and often obstructed observation and procurement of released sperm. Female-acting individuals sometimes glide during intromissions, decreasing efficiency of extraction.

4.6 Future Research

4.6.1 Concentration

A priority in any future research would be to establish the optimal centrifugation time at which all sperm form one pellet. Rogers and Chase (2001) used centrifuged sperm transferred in spermatophores of the terrestrial snail *Helix aspersa*. at 4,000 rpm for 5min to form a pellet, which was air-dried then resuspended before counting on a Neubaurer haemocytometer (Rogers & Chase 2001).

4.6.2 Sperm Counting

After centrifugation to form a pellet, excess water should be removed from sperm samples and the pellet resuspended. Recommended methods for sperm resuspension are a thermoshaker or sonicator, which resuspend pellets without applying excessive force that could potentially damage sperm. Locher and Barr (1997) used a sonicator to break up sperm clumps and counted sperm number in the

simultaneously hermaphroditic terrestrial snail *A. arbustorum*. However individuals were killed and sperm clumps obtained from spermatophores (Locher & Barr 1997).

4.6.3 Surface area estimation

Surface area estimates of sperm samples pelleted by centrifugation are also of potential interest.

5. Conclusion

This is the first study to investigate a method of measuring sperm transfer in *Chelidonura sandrana*. This project investigated methodological tools for future experimentation relating copulation duration to sperm transferred in *C. sandrana*. Low concentrations of free sperm and sperm clustering both were challenges in this experiment at various times. Improving centrifugation methods would allow counts by surface area estimation. A method to resuspend sperm pellets would enale free sperm counts. In conclusion, this study provides a background for future work measuring sperm transfer in *C. sandrana*.

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