พฤติกรรมการผสมพันธุ์ของหมึกกระดองลายเสือ *Sepia pharaonis*

นายแทนไท ประเสริฐกุล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาวิทยาศาสตร์ทางทะเล ภาควิชาวิทยาศาสตร์ทางทะเล
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2549

MATING BEHAVIORS OF PHARAOH CUTTLEFISH *Sepia pharaonis*

Mr. Tanthai Prasertkul

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Marine Science
Department of Marine Science
Faculty of Science
Chulalongkorn University
Academic Year 2006

Thesis Title	MATING BEHAVIORS OF PHARAOH CUTTLEFISH *Sepia pharaonis*
By	Tanthai Prasertkul
Department	Marine Science
Thesis Advisor	Assistant Professor Charoen Nitithamyong, Ph.D.
Thesis Co-advisor	Sanit Piyapattanakorn, Ph.D.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

..... Dean of the Faculty of Science

(Professor Piamsak Menasveta, Ph.D.)

THESIS COMMITTEE

..... Chairman

(Associate Professor Somkiat Piyatiratitivorakul, Ph.D.)

..... Thesis Advisor

(Assistant Professor Charoen Nitithamyong, Ph.D.)

..... Thesis Co-advisor

(Sanit Piyapattanakorn, Ph.D.)

..... Member

(Assistant Professor Thaithaworn Lirdwitayaprasit, Ph.D.)

..... Member

(Assistant Professor Suchana Chavanich, Ph.D.)

แทนไท ประเสริฐกุล: พฤติกรรมการผสมพันธุ์ของหมึกกระดองลายเสือ *Sepia pharaonis*. (MATING BEHAVIORS OF PHARAOH CUTTLEFISH *Sepia pharaonis*) อ. ที่ปรึกษา: ผศ. ดร. เจริญ นิติธรรมยง, อ.ที่ปรึกษาร่วม: ดร. ศานิต ปิยพัฒนากร, 118 หน้า.

การศึกษาครั้งนี้ มีจุดประสงค์เพื่อสำรวจและรายงานรายละเอียดพฤติกรรมการผสมพันธุ์ในหมึกกระดองลายเสือ *S. pharaonis* โดยมุ่งเน้นที่พฤติกรรมของตัวผู้ซึ่งเกี่ยวข้องกับการแข่งขันเพื่อเพิ่มโอกาสในการปฏิสนธิให้กับสเปิร์มของตนเอง (sperm competition) เช่น พฤติกรรมการกำจัดสเปิร์มของคู่แข่ง (sperm removal) การอยู่เฝ้าป้องกันคู่ผสม (mate guarding) และการผสมซ้ำ (re-copulation) ผลการศึกษาในห้องทดลองพบว่า หมึกกระดองลายเสือผสมพันธุ์ในตำแหน่งหันหัวเข้าหากัน การผสมพันธุ์ใช้เวลาเฉลี่ย 10 นาที โดยตัวผู้ถ่ายเทฝักสเปิร์มไปติดไว้กับแผ่นเยื่อบริเวณใต้ปากของตัวเมีย ในกรณีที่ผสมพันธุ์กับตัวเมียที่ได้รับการผสมพันธุ์มาก่อน ตัวผู้ใช้ด้านที่เป็นถ้วยดูดของหนวดคู่ที่ 3 ขูดฝักสเปิร์มที่ตัวผู้ตัวอื่นได้ติดไว้ออกมาก่อน จากนั้นจึงถ่ายเทฝักสเปิร์มของตนเอง การกำจัดฝักสเปิร์มใช้เวลาเฉลี่ย 1.5 นาที จำนวนฝักสเปิร์มที่ถูกกำจัดออก คิดเป็นร้อยละ 34 ของจำนวนฝักทั้งหมดที่ตัวผู้ตัวแรกได้ติดเอาไว้ ตัวผู้ใช้หนวดคู่ที่ 4 ข้างซ้ายในการถ่ายเทฝักสเปิร์ม และจะถ่ายเท 2 ครั้งติดต่อกัน ก่อนจะหยุดไปประมาณ 1.5 นาทีเพื่อติดฝักสเปิร์มให้เข้าที่ จากนั้นจึงถ่ายเทต่ออีก 2 ครั้งติดต่อกัน เช่นนี้ไปเรื่อยๆ ในการผสม 1 รอบ มีการถ่ายเททั้งหมด 8 ถึง 10 ครั้ง แต่ละครั้งถ่ายเทฝักสเปิร์มประมาณ 9 ฝัก และเมื่อผสมเสร็จแล้วจะติดฝักสเปิร์มไว้กับเยื่อใต้ปากของตัวเมียรวมทั้งสิ้นประมาณ 60 ฝัก ตัวเมียเมื่อได้รับการผสมจากตัวผู้ 2 ตัว จะมีฝักสเปิร์มติดอยู่ที่เยื่อใต้ปากทั้งหมดประมาณ 100 ฝัก โดยในจำนวนนี้จะเป็นของตัวผู้ที่ผสมตัวแรกร้อยละ 43 และของตัวผู้ที่ผสมทีหลังร้อยละ 57 ตัวผู้ส่วนใหญ่เมื่อผสมเสร็จแล้ว จะอยู่เฝ้าติดตามตัวเมียอย่างใกล้ชิด และหลังจากเวลาผ่านไปประมาณ 50 นาที จะทำการผสมซ้ำกับตัวเมียตัวเดิมอีกรอบ ถึงแม้ว่าจะมีตัวเมียตัวอื่นอยู่ด้วยก็ตาม

ภาควิชา วิทยาศาสตร์ทางทะเล..... ลายมือชื่อนิสิต.....

สาขาวิชา วิทยาศาสตร์ทางทะเล..... ลายมือชื่ออาจารย์ที่ปรึกษา.....

ปีการศึกษา 2549..... ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

467 22706 23: MAJOR MARINE SCIENCE

KEY WORD: SPERM COMPETITION / SPERM REMOVAL / MATING BEHAVIOR / CUTTLEFISH

TANTHAI PRASERTKUL: MATING BEHAVIORS OF PHARAOH CUTTLEFISH *Sepia pharaonis*. THESIS ADVISOR: ASST. PROF. CHAROEN NITITHAMYONG, Ph.D., THESIS COADVISOR: SANIT PIYAPATTANAKORN, Ph.D., 118 pp.

This study aimed to elucidate details of mating behaviors in the pharaoh cuttlefish *S. pharaonis*, with an emphasis on male behavioral adaptations that are related to sperm competition, such as sperm removal, mate guarding, and re-copulation. Observations in the laboratory showed that copulations occurred in the head-to-head position, and lasted about 10 minutes. When copulating with a recently mated female, the male exhibited sperm removal behavior, by using the sucker side of his third arms to scrape off spematangia deposited during previous mating. The male spent the first 1.5 minutes of copulation to remove on average 34% of previous male's spermatangia before transferring his own to the ventral region of the female's buccal membrane, using the hectocotylized 4th left arm. Spermatophore transfers occurred in sets of two closely consecutive transfers, each set separated by 1.5 minutes of placement phase. The whole copulation session consisted of 8-10 transfers. Nine spermatophores were passed during each transfer, and a total of about 60 were successfully deposited during a single copulation. After mating with 2 males, the female's buccal membrane had about 100 spermatangia attached to it. Of these, 43% belonged to the first male, and 57% belonged to the second male. Most males guarded the female closely after copulation, and after about 50 minutes, re-copulated with her again regardless of the presence of another available female.

Department	Marine Science.....	Student's signature.....
Field of study	Marine Science.....	Advisor's signature.....
Academic year	2006.....	Co-advisor's signature.....

ACKNOWLEDGEMENTS

I would like to thank my advisor, Assistant Professor Dr. Charoen Nitithamyong, and my co-advisor, Dr. Sanit Piyapattanakorn for their supervision on this thesis. Thank you to Dr. Renu Yashiro, the director of Rayong Coastal Fisheries Research and Development Center, for providing the facility needed to conduct the study. Thank you Associate Professor Dr. Orawan Satayalai for the advice on vital dyes and staining techniques. Thank you Dr. Jaruwat Nabhitabhata for his pioneering works in *S. pharaonis*. Sincere thanks to Associate Professor Dr. Somkiat Piyatiratitivorakul, Assistant Professor Dr. Suchana Chavanich, Assistant Professor Dr. Thaithaworn Lirdwitayaprasit, Associate Professor Nittharat Paphavasit, Assistant Professor Dr. Ajcharaporn Piumsomboon, Dr. Marie-Jose Nuad, Dr. Jean Boal, Dr. Paul Shaw, and Dr. Roger Hanlon for their comments, suggestions, and supports, which have been essential for the completion of this study. Thank you very much to Mr. Somtob Wachsomrit for his assistance throughout all the experiments. This study could not have been finished without his help. Also, thanks to all the people in Dr. Sanit's laboratory for helping me with the molecular methods. And finally, thank you so much to my family and my loved ones for their patience and supports. Two and a half years of working on this thesis has not been easy, and it is good to know there are still people behind me who always care, appreciate, and understand.

TABLE OF CONTENTS

Page

ABSTRACT (THAI)……iv

ABSTRACT (ENGLISH)……v

ACKNOWLEDGEMENTS……vi

TABLE OF CONTENTS……vii

LIST OF TABLES……x

LIST OF FIGURES……xi

CHAPTER I INTRODUCTION……1

1.1 Sperm competition……1

1.1.1 Sperm removal……2

1.2 General features of mating behaviors and sperm competition in cuttlefish……4

1.3 Mating behaviors and sperm competition in the pharaoh cuttlefish *Sepia pharaonis*……10

1.4 Objectives of the current study……11

CHAPTER II MATERIALS AND METHODS……13

2.1 Experiment I……13

2.1.1 Animals and husbandry conditions……13

2.1.2 Observation and analysis of mating behaviors……16

2.1.3 Quantitative and statistical analysis……23

2.2 Experiment II……26

2.2.1 Animals and husbandry conditions……26

2.2.2 Mating trials and the resulting eggs……27

2.2.3 Tissue sampling and DNA extraction……29

2.2.4 ISSR-PCR and primer screening……31

2.2.5 Statistical analysis……32

2.3 Experiment III……33

Page

2.3.1 Animals and husbandry conditions 33
2.3.2 Testing re-copulation.....33
2.3.3 Observation of pre- and post-copulatory behaviors.....35
2.3.4 Statistical analysis 36

CHAPTER III RESULTS.....37
3.1 Experiment I.....37
3.1.1 The male reproductive anatomy.....37
3.1.2 Spermatophore, spermatangia, and the mechanism of ejaculation.....42
3.1.3 Female buccal membrane and sperm receptacles 46
3.1.4 Details of copulation.....48
3.1.4.1 The sperm removal phase 48
3.1.4.2 Spermatophore transfer 52
3.1.4.3 Spermatangia placement phase.....54
3.1.4.4 Overall pattern of copulation sequence.....57
3.1.4.5 Number of spermatophore transfers per mating.....59
3.1.4.6 Number of spermatophores transferred per mating 59
3.1.4.7 Copulation duration.....60
3.1.5 Factors affecting sperm removal.....62
3.1.6 Sperm removal by the female.....67
3.1.7 Analysis of spermatangia attached on the female's buccal membrane.....67
3.2 Experiment II.....70
3.2.1 Mating duration 70
3.2.2 Fecundity and hatching rate.....71
3.2.3 ISSR-PCR primer screening 74
3.3 Experiment III.....75
3.3.1 Mating and re-copulation 75
3.3.2 Pre-copulatory behaviors 79
3.3.2.1 Female rejection.....79
3.3.2.2 Male courtship and initiation of copulation.....81

Page

3.3.3 Post-copulatory behaviors....89

CHAPTER IV DISSCUSIONS....92

4.1 Sperm removal....92

4.2 Mechanism of sperm removal....93

4.3 Extent of sperm removal....94

4.4 Factors related to variations in the amount of sperm removed and the duration of the removal phase....97

4.5 The attachment-space hypothesis....100

4.6 Removal of self-sperm....101

4.7 Comparative features of spermatophore transfer and copulatory sequence....102

4.8 Mating duration....104

4.9 Conclusions....106

REFERENCES....107

APPENDICES....114

Appendix A....115

Appendix B....117

BIOGRAPHY....118

LIST OF TABLES

Page

Table 2.1 Mating scheme of experiment I.. 17

Table 2.2 Mating scheme of experiment II. 28

Table 2.4 Mating scheme of experiment III. 35

Table 3.1 Duration of 1st and 2nd mating, and the duration of interval that elapsed between them. 71

Table 3.2 The number of eggs contained in each clutch, the number of eggs hatched, the incubation period, hatching rate, and the number of days between spawnings. 73

Table 3.3 ISSR primers selected for testing DNA fingerprints for each family. 75

Table 3.4 Mating results of trials in experiment III.. 76

Table 3.5 Results of trials that used the same male, paired with the same two females, but conducted on different dates. 79

Table 3.6 Signal components of female rejection in *S. pharaonis* 80

Table 3.7 Signal components of The Round Face Tiger Display in *S. pharaonis* 81

Table 3.8 Signal components of the Buffalo Display in *S. pharaonis* 84

Table 3.9 Signal components of the Intermediate Display in *S. pharaonis* 86

Table 3.10 Different categories of pre-copulatory behaviors observed in trials that mating occurred at least once 88

Table 3.11 Different categories of behaviors observed during the period between the first and the second copulation, in trials that re-copulation occurred with the same female. 90

Table A.1 Names of ISSR primers used during screening and their 5' to 3' oligonucleotide sequences 115

LIST OF FIGURES

Page

Fig. 1.1 a) Two *S.Officinalis* males showing the Intense Zebra Display while engaging in agonistic interactions. b) Male and female *S.Officinalis* copulating in the head-to-head position. 4

Fig. 1.2 Basic terms and structures that are commonly referred to in cuttlefish. a) Schematic illustration of a cuttlefish showing how mantle length is defined. b) Oral view, the buccal membrane, and the roman numerals designated to each arm. c) Ventral view, the arms, the tentacles, and the funnel. d) The hectocotylized left 4th arm of the male.. 6

Fig. 1.3 Characteristics of the pharaoh cuttlefish, *S. pharaonis*. 11

Fig. 2.1 The cement tanks used to keep the animals. a) Water supply. b) Lighting scheme during day time. c) Fluorescent light provided at night during the experiment. 14

Fig. 2.2 Sexually dimorphic characteristics in *S. pharaonis*. a) Two males showing the Intense Zebra Display toward each other. b) The tiger pattern on the mantle and the arms of the male. c) A male and a female. 15

Fig. 2.3 a) and b) The experimental tank with transparent bottom, through which mating could be observed. c) The camera's ventral field of view captured movements of the arms and the funnel during mating. d) A 400W spot light used to lit the tank from below during mating. 18

Fig. 2.4 a) Ventral view of a female's mouth area. b) Close up of the female's ventral buccal membrane with numerous attached spermatangia.....18

Fig. 2.5 a) Anaesthetized female in 1-2% ethanol. b) and c) Application of dye to the attached spermatangia using a paint brush. d) The spermatangia attached by the second male (white) and those that remained from the first mating (purple). 19

Fig. 2.6 a) Different kinds of material that fell to the floor of the tank during mating. b) Close up of colored spermatangia. 21

Page

Fig. 2.7 a) The dissected female's ventral buccal membrane b) Spermatangia deposited from the first mating (purple) and from the second mating (white). c) and d) Using a forceps to pull spermatangia out one by one until none was left on the membrane. 23

Fig. 2.8 Dividers made of wooden frames and plastic nettings used to separate individuals living in the same tank. 27

Fig. 2.9 a) and b) The egg mass attached to the wooden mesh. c) and d) Several days old eggs ripped from the mesh one by one and were put in a floating basket during incubation period. 29

Fig. 2.10 a) Eggs ready to hatch. b) Hatchlings about 0.5-1 cm long. c) Hatchlings collected and put in a plastic jar filled with absolute ethanol. 30

Fig. 2.11 Two fluorescent lights positioned at the edge of the tank during the filming of each mating trial. 34

Fig. 3.1 Male reproductive anatomy. a) Ventral view of internal organs, showing the Needham's organ, protruding from underneath the base of the left gill. b) After all the connective tissues have been cut, the whole reproductive-organ complex can be separated as a package. c) Close up of the reproductive-organ complex showing, the testis, the coiled vas deferens, the Needham's sac, and the spermatophores tightly packed inside. 38

Fig. 3.2 Relationship between the body size of the male and the size of the Needham's organ. 39

Fig. 3.3 a) Normal, fully developed spermatophores tightly packed inside the Needham's organ. b) and c) Spermatophores that are still in various stages of development. d) Bursted, degenerated, abnormal spermatophores found in one male several hours after death. 40

Fig. 3.4 a) A male's left fourth arm with reduced suckers, or the hectocotylized portion at the base. b) The right fourth arm of the same male with the hectocotylized portion absent. 41

Page

Fig. 3.5 a) Illustration of a typical cephalopod spermatophore showing different components. b) Close up of the cap region showing the cement body, the coiled ejaculatory apparatus, the cap, and the thread. c) When spermatophore is evaginated, the sperm mass becomes exposed and is re-termed a spermatangium. 43

Fig. 3.6 a) and b) Intact spermatophores of *S. pharaonis*, 1.5-2 cm long. c) and d) Spermatophores with thin threads connecting from the caps to the Needham's organ. e) and f) The base of the sperm mass, the cement body, the ejaculatory apparatus, and the cap. 44

Fig. 3.7 a) to h) Chronological series of video captures showing the process of spermatophore evagination. 45

Fig. 3.8 a) and b) Newly emerged spermatangia (sickle shape), Some still attached to the spermatophore sheaths. c) Sample of white mass inside spermatangia was found to contain numerous live sperm. d) Spermatangia that were found attached to the ventral region of the female's buccal membrane after mating. 46

Fig. 3.9 a) Ventral view of a female showing the paired seminal receptacle inside the buccal membrane. b) The other side of the buccal membrane reveals attached spermatangia. c) Close up of the receptacles in a dissected buccal membrane showing clusters of glandular sacs that are used to store sperm. d) On the opposite side, there are 2 openings that lead to the receptacles. 47

Fig. 3.10 Video captures of copulation from the ventral view during the sperm removal phase. a) The male used the third pair of arms to press and scrape against the female's buccal area. b) Arrows indicate the removed purple spermatangia falling to the floor. c) An illustration of sperm removal behavior from a lateral view. 49

Fig. 3.11 Removed spermatangia contained the purple color. 51

Fig. 3.12 a) to d) Chronological series of video captures showing the process of spermatophore transfer from a ventral view. 53

Page

Fig. 3.13 a) Spermatangia placed on the inner surface of the ventral buccal membrane. b) Some spermatangia placed on the arms. 54

Fig. 3.14 Relationship between a) The average duration of the placement phase and the overall duration of the copulation. b) The average duration of placement phase and the number of spermatophore transfers during that copulation. 56

Fig. 3.15 a) Empty spermatophore sheaths being blown out from the mouth area during mating. b) Spermatophore sheaths and other debris that have settled to the floor of the tank after mating.....57

Fig. 3.16 A typical copulation sequence of *S. pharaonis*, ...58

Fig. 3.17 Relationship between the number of transfers and the number of spermatophores transferred per copulation. 60

Fig. 3.18 Relationship between a) copulation duration and the number of transfers. b) copulation duration and the number of spermatophores transferred. 61

Fig. 3.19 Relationship between the number of spermatangia attached by the first male and a) the number of spermatangia removed by the second male, and b) the percentage of attached spermatangia that are removed.....63

Fig. 3.20 Relationship between duration of the removal phase and the number of spermatangia removed.....64

Fig. 3.21 Relationship between: a) Duration of the removal phase and the copulation duration (1st mating). b) Duration of the removal phase and the average duration of placement phase during the same copulation (1st mating).....65

Fig. 3.22 Relationship between: a) Duration of the removal phase and the copulation duration (2nd mating). b) Duration of the removal phase and the average duration of placement phase during the same copulation (2nd mating).....66

Page

Fig. 3.23 Percentage of spermatangia that resulted in different fates. a) The 2nd male's spermatangia. b) The 1st male's spermatangia. 69

Fig. 3.24 Whole spermatangia were sometimes found hidden inside the cavity of seminal receptacle.....70

Fig. 3.24 Relationship between duration of the first copulation and a) the time elapsed until the second copulation (interval), and b) duration of second copulation. 77

Fig. 3.25 Relationship between the interval and second mating's duration.....78

Fig. 3.26 a) to d) Female rejection in *S. pharaonis*.. 80

Fig. 3.27 a) to c)The male's Round Face Tiger Display in *S. pharaonis*.....82

Fig. 3.28 Two *S. pharaonis* males engaging in agonistic interactions, showing off their Intense Zebra Display 83

Fig. 3.29 The Buffalo Display in male *S. pharaonis*. 84

Fig. 3.30 Copulation of *S. pharaonis* in the head-to-head position.....85

Fig. 3.31 The Intermediate Display, exhibited by male *S. pharaonis* 87

Fig. 3.32 Post-copulatory mate guarding in *S. pharaonis*. a) The male resting his arms on her dorsal surface. b) The male showing aggressive Intense Zebra Display toward the other female whom he had not mated with. 89

Fig. B.1 Examples of ISSR profiles of *S. pharaonis* individuals 117

CHAPTER I

INTRODUCTION

1.1 Sperm competition (Parker, 1970)

In species whose females exhibit multiple mating with different males, and possess the capability to store sperm for a certain period of time, there is a possibility that the sperm from different males can overlap temporally and spatially inside the female reproductive tract or at the site of fertilization. Whenever such conditions are met, sperm competition inevitably occurs. It is defined as the competition between sperm of two or more males for the fertilization of a given set of ova. Sperm competition is an important force in the process of sexual selection, which could have profound influences on the evolution of male reproductive morphology, physiology, and behavior. It generates a selection pressure favoring adaptations in males that help raise the chance of fertilization by self sperm over that of the rival. Such adaptations may function by preempting the sperm stored from previous mating (e.g. sperm removal), thus allowing males to gain the majority of fertilization when mating with previously mated females. On the other hand, there are also adaptations that prevent any future males from mating with the female (e.g. mate guarding), thus enabling the current male to avoid or reduce subsequent competition from the sperm of another male. More generally, the male can also increase its chance of fathering offspring by inseminating the female with as much sperm as possible, so that they outnumber those of the rival (e.g. multiple re-copulations with the same female). Sperm competition and its evolutionary consequences have given rise to a wide variety of adaptations that are widespread among the males of many species (see review by Birkhead & Møller, 1998; Simmons, 2001). Sperm removal is one of such adaptations that have been commonly demonstrated across taxa, and is the main focus of the current study.

1.1.1 Sperm removal

Sperm removal occurs when the copulating male removes sperm of rival males previously deposited in the female's sperm storage organ, before transferring his own sperm. By doing so, the male increases the relative number of self's sperm in the storage site, and thereby enhances the chance of fathering the offspring. Sperm removal has been well demonstrated in insects such as damselflies and dragonflies (order Odonata), whose males possess specially modified penis, equipped at the tip with horns, hairs, or backward facing spines, which the male utilize in various ways to physically remove the stored sperm (Waage, 1979; Waage, 1986; Miller, 1987; Siva-Jothy, 1987; Córdoba-Aguilar, 2003; Córdoba-Aguilar et al., 2003). In the yellow mealworm beetle, and the flour beetle (order Coleoptera), the male genitalia also have spines that can remove stored sperm (Gage, 1992; Haubruge at al., 1999). In earwigs (order Dermaptera), the male inserts one of the paired intromittent organs, which is as long as his body, deep into the female's spermatheca, then extracts it while ejaculating, and simultaneously removing rival sperm using a fringe-like projection on the penis's tip (Kamimura, 2000; Kamimura, 2005). In the bushcricket (order Orthoptera), the male has been shown to eliminate rival sperm, not by direct removal, but by using his subgenital plate to stimulate the female's spermathecal openning, mimicking the passing through of eggs during oviposition, and thereby inducing the female to release stored sperm by mechanical reflex (Helversen & Helverson, 1991). In another orthopteran, the tree cricket, the mating male uses his own ejaculate to force around 90% of rival sperm out of storage and onto his own genitalia for subsequent removal and ingestion (Ono et al., 1989). Males of dung flies similarly use their own ejaculate to dilute and displace the sperm of previous males in storage (Parker & Simmons, 1991).

Sperm removal has also been revealed in few other groups of animals besides the insects. Among the crustaceans, males of crayfish (Astacidae) have been shown to eat all or most of spermatohores previously deposited by other males before releasing their own sperm (Reynolds et al., 1992; Villanelli & Gherardi, 1998; Galeotti et al., 2007).

In some species of birds, such as the Dunnock, males have been observed to peck at the cloaca of the female, causing her to eject a droplet of sperm presumably deposited by previous males (Davies, 1983). This behavior is called “cloaca pecking” or “cloaca inspection”, and is found in various other species of birds as well. In humming birds, it has been proposed that the male may be able to use its long tongue to remove sperm directly from inside the female’s reproductive tract (Leonard, 2001). In Argentine lake ducks, the penis of a male is over 20 cm long and is covered with coarse spines at the base and soft brush-like projections at the tip, which have been postulated to function in sperm removal (McCracken, 2000). In many reptiles (lizards, snakes) and mammals (rodents, cats, bats, primates), the surface of the penis is also decorated with spines (Eberhard, 1985; Ryan, 1991; Harcourt & Gardiner, 1994; Stockley, 2002; Parag et al., 2006). Many hypotheses regarding the functions of these penal spines have been proposed, including possible roles in locking the mating pair together, or stimulating the female to enhance her reproductive readiness and enhance sperm uptake. Their possible roles in sperm removal, however, have not received much attention so far. In human, males possess a longer penis with wider diameter, larger glans, and more pronounced coronal ridge when compared in relation to other primates. Some authors have suggested that these features, combined with repeated thrusting before ejaculation, may function to remove foreign semen by drawing it back and away from the cervix (Baker & Bellis, 1995; Gallup, 2004).

In cuttlefish (mollusca: cephalopoda: sepia), the subject of this study, females exhibit both sperm-storage capability and multiple mating with multiple partners, thus representing an interesting model system, in which the adaptations to sperm competition including sperm removal can be expected. In addition, male cuttlefish copulates by attaching numerous spermatangia on the membrane underneath the female’s mouth (the ventral buccal membrane), which is an external site that other males should have an easy access to, making it very likely that sperm removal may have evolved in this group. So far, only a few studies have investigated mating behaviors of cuttlefish in the context of sperm competition, or sperm removal (Hanlon et al., 1999;

Hall & Hanlon, 2002; Nuad et al., 2004; Wada et al., 2005). Some suggested jetting of water through the siphon or “flushing” as a mean for removing rival sperm (Hanlon et al., 1999; Hall & Hanlon, 2002), whereas some suggested that the males may use the teeth on the suckers of their arms to scrape off rival sperm mass in the same manner that the spines on the damselflies’ penis are used for (Wada et al., 2005). The following section describes in more details about the existing knowledge on general features of mating behaviors in different species of cuttlefish, and the adaptations that may have arisen in response to sperm competition.

1.2 General features of mating behaviors and sperm competition in cuttlefish

Among the cuttlefish of genus *Sepia*, mating and sexual behaviors have been studied primarily in *S. officinalis* (Boal, 1996; Boal, 1997; Adamo & Hanlon, 1996; Hanlon et al., 1999), *S. apama* (Hall & Hanlon, 2002; Nuad et al., 2004; Nuad et al., 2005), *S. latimanus* (Hanlon & Messenger, 1996), and *S. esculenta* (Wada et al., 2005). General behavioral features shared among different species can be drawn from the results of these studies, and can be summarized as follows. First, while competing for females, the males often engage in agonistic contest by putting on a distinct display called the Intense Zebra Display (Fig. 1.1a) toward one another. If neither one retreats, then the

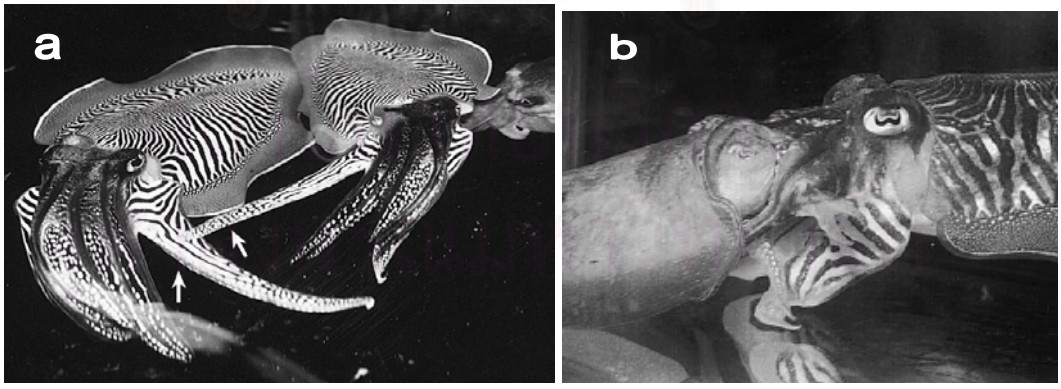


Fig. 1.1 a) Two *S.Officinalis* males showing the Intense Zebra Display while engaging in agonistic interactions (Source: Hanlon et al., 1999). b) Male (right) and female (left) *S.Officinalis* copulating in the head-to-head position (Source: Adamo & Hanlon, 1996).

contest may escalate to the level of physical fighting, until one of them is defeated. Once the male gains access to a female, he approaches her and initiates courtship by swimming parallel to her. During this period, males of different species often exhibit their own distinct courtship colorations and stripe patterns. The female may show acceptance to the male by remaining still, or she may respond negatively by jetting away and putting on a distinctive rejection display, which could be different for each species. If the female do not resist, the male then proceed to use his arms to touch gently on the female's mantle, head, and arms, while hovering closely above or alongside her. Eventually, the male grasps the female's arms with his arms and brings her toward the 'head-to-head' position (Fig. 1.1b), in which the couple starts to copulate. During copulation, the male uses its modified left fourth arm, termed the hectocotylus (Fig. 1.2d), to pass spermatophores to the ventral buccal membrane of the female (Fig. 1.2b,c), where a pair of seminal receptacles (the sperm-storage organ) is located. In cuttlefish, each spermatophore has an outer sheath that is discarded during mating. Only the sperm mass, or the spermatangium that is inside the sheath gets attached to the female's buccal membrane.

In *S. officinalis*, the male typically transfers spermatophores once, and deposits a total of about 130-150 spermatangia on the female's buccal membrane during a single copulation (Hanlon et al., 1999). Mating in this species can last up to 20 minutes, but normally takes 2-5 minutes in the laboratory (Hanlon, 1996). A single pair can mate several times in succession. Egg laying occurs between these successive matings in some occasions. Both sexes accept multiple mates, and temporary mate guarding by the male have been observed in the laboratory. While copulating, males use movement of their mantles to rhythmically and vigorously pump large quantities of water through their funnels into the female's buccal area. Hanlon et al. (1999) were the first to study this "flushing" behavior in the context of sperm competition, and to address it as a

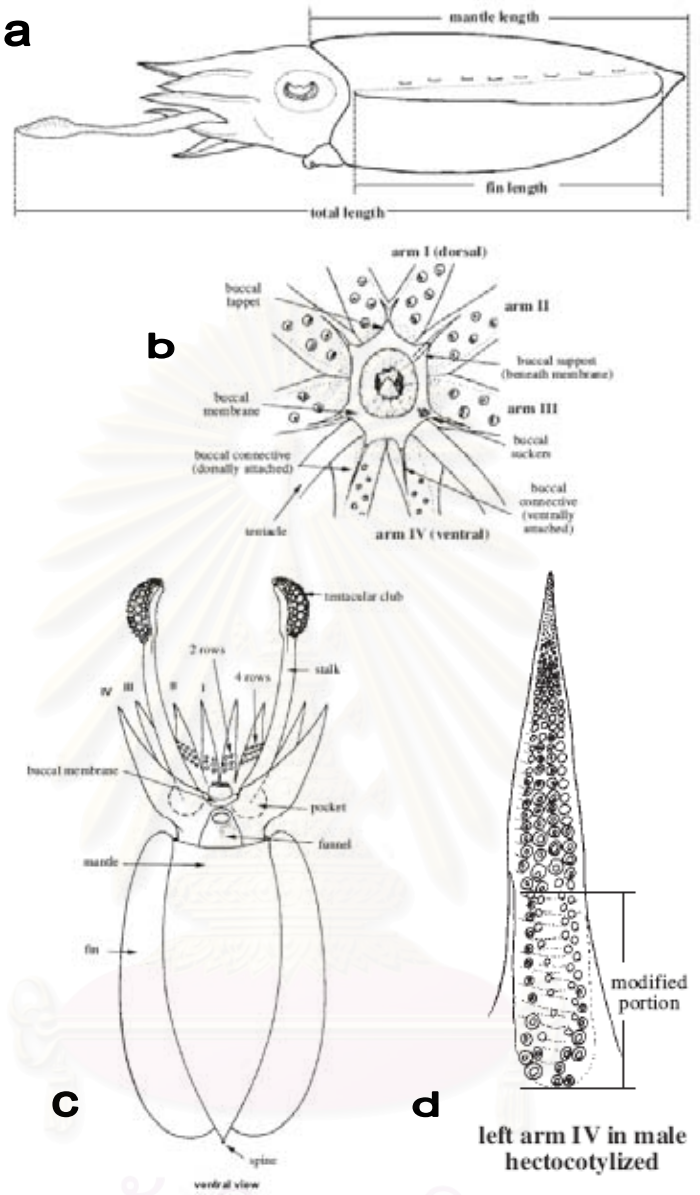


Fig. 1.2 Basic terms and structures that are commonly referred to in cuttlefish. a) Schematic illustration of a cuttlefish showing how mantle length is defined. b) Oral view with the mouth at the center showing the buccal membrane, and the roman numerals designated to each arm. c) Ventral view showing the arms, the tentacles, and the funnel or siphon. d) Only the left 4th arm of the male has a specially modified portion with reduced suckers. The structure is called the hectocotylus, and it is used during mating for handling and transferring spermatophores. Images are modified from Jereb & Roper, 2005.

possible mechanism by which the males might utilize to remove sperm of others. The force of water jets that the copulating male pumps toward the female's buccal region may cause spermatangia deposited there by other males from recent previous matings to be detached. Hence, by doing so, the copulating male reduces the competition with other males' sperm, and increases the likelihood of its own sperm to fertilize the eggs.

The result of the study (Hanlon et al., 1999) found that approximately males spend the first 6 minutes of copulation, or 63% of the total mating period, engaging in vigorous flushing action. When females are mated first by one male and then immediately afterwards by the second, small white bundles can be seen falling out, as the second male start to flush water toward the female's buccal area. Hanlon et al. (1999) suggested that these bundles were pieces of spermatangia from previous mating since the current male had not yet transferred its own spermatophores. However, such obvious removal of spermatangia from previous matings was only observed when the second copulation occurred almost immediately after the first. When females were mated a day or two after a previous mating, there was no visible material of any kind being released during the male's flushing actions. This suggests that the male's flushing behavior can serve as a mean to remove rival sperm, but its effectiveness may be restricted to only a short period of time right after the previous copulation.

In *S. esculenta*, Wada et al. (2005) found a different sperm removal behavior. The males of this species do not use flushing, but instead, use 'scraping' as a mean to remove rival sperm. Mating starts off in the head-to-head position, and during the first phase, termed the sperm removal phase (lasted on average 93 seconds), the males use the sucker side on both of their third arms to scrape off sperm masses attached on the ventral region of female buccal membrane during previous mating. During this process much debris is generated and released into the water. The particles have been verified to be pieces of sperm mass that fall out of the female's buccal cavity. After the sperm removal phase, many sperm masses still remain attached on the female buccal membrane, showing that scraping behavior could not remove all of them. The numbers

of particles of sperm mass that are successfully removed correlate positively with the amount of time the male spends in the removal phase. Interestingly, the male performs sperm removal in every observed matings, even when he himself has just mated with the female (therefore removing his own sperm). Moreover, the duration of sperm removal does not differ significantly whether the last male to mate with the female is the removing male or a different male. This suggests that the male does not discriminate between removing rival sperm and removing the sperm that he himself deposits.

Hall and Hanlon (2002) conducted a field study in *S. apama*, which forms a large (thousands of individuals) annual spawning aggregation over a rocky reef area in South Australia. The sex ratio is highly biased toward males during the spawning season (average 4 males to 1 female). Females are observed to mate with multiple males (up to 4 different males before laying an egg), potentially accumulating sperm from consecutive matings, and setting the stage for intense sperm competition. Similar to *S. officinalis*, males spend the first 71% of the mating time engaging in flushing behavior. However, the researchers also noted that flushing was accompanied by movements of the male's second and third arms into the buccal area of the female, which is similar to what was found in *S. esculenta* (Wada et al., 2005). Large males spend a greater percentage (almost 80%) of mating time in the flushing stage compared to small males (about 60%). Other than this, size classes seem to play a more important part in other aspects of competition rather than the actual details of copulation. Large males are more successful at pairing with females, and at defending mates from other challenging males. Small males often adopt "sneaker" tactics, in which they do not challenge paired males directly, but instead attempt to sneak up and mate with the female while the paired male was being distracted (copulation achieved outside of paring in this manner is called "Extra Pair Copulation" or EPC). By this, and also by winning some direct challenges, small males are able to achieve almost half of the successful matings observed. However, mating success may not necessarily translate into fertilization success due to post-copulatory sperm competition mechanisms, such as mate guarding. Large paired males seem to prioritize fighting against large male challengers

over small males or over guarding the females. For example, on some occasion large paired males and large challengers would stay fixed in agonistic contests for minutes, leaving the female more exposed to EPC attempts by small sneaker-males. This suggests that large males posed greater threat than small males in terms of sperm competition.

Nuad et al. (2004) conducted a field study on the same *S. apama* population and found approximately the same behavioral patterns as Hall and Hanlon (2002). However, in addition, Nuad et al. (2004) utilized microsatellite DNA analysis to determine paternities of sampled eggs, and to establish a link between each individual male's behavior and his reproductive success. The result showed that eggs sampled from 6 out of 9 females were indeed fertilized by multiple males. Since flushing behavior is thought to be the mechanism used for sperm removal, the last male to mate is therefore expected to have a greater chance in siring offspring than the previous males whose spermatangia has been partially removed (a pattern termed "last-male sperm precedence"). This prediction was not significantly supported by the result of the study (however, paternity by the last male was shown in 50% of the cases, indicating that all previous males must share the remaining 50% success). Also, males that spent more time flushing did not gain more fertilization success. This suggests that flushing may not serve to remove spermatangia from earlier matings as previously predicted. However, it is still possible that the effectiveness of flushing may depend on many factors (such as time elapsed since previous matings), and thus, could not be measured based on its duration alone. In other words, the amount of time a male spent in flushing may not accurately reflect the amount of rival sperm he actually managed to remove. The result did show a correlation between fertilization success and the time that the egg was laid after mating. Eggs laid 20-40 minutes after mating with a particular male are more often fertilized by that male than those laid before or after this time. No significant difference was found between the reproductive success rates of males from different statuses (with a pair vs. without a pair), or size classes (large vs. small). This is consistent with data from previous study (Hall & Hanlon, 2002) which had shown that small males adopt

various alternative sneaking tactics, and were quite successful at obtaining copulations without having to actually form pairs with females.

1.3 Mating behaviors and sperm competition in the pharaoh cuttlefish *Sepia pharaonis*

S. pharaonis (Fig. 1.3) is an economically important species found in the Gulf of Thailand and the Andaman Sea from the coastal shallows to about 100 m depth, with most caught between 10 and 40 m (Jereb & Roper, 2005). Mating behaviors of this species have only been mentioned briefly in studies that primarily aimed toward developing techniques for large-scale commercial cultivation (Nabhitabhata, 1995; Nabhitabhata & Nilaphat, 1999; Minton et al. 2001). During mating in captivity, a male approaches a female and displays tiger color pattern, with his first pair of arms raised up. If the female accepts, the male touches her with his arms and then form a swimming pair with her. To begin copulation, the male hovers above the female in a parallel position, and starts to drape his arms around her dorsal surface. He then grasps her by his arms, turns his body around into the head-to-head position, and begins to copulate. Spermatophores are transferred by the fourth arm of the male, and fixed to the seminal receptacle in the buccal region of the female. The durations of copulation vary from less than 1 minute to more than 30 minutes. The male releases the female afterwards, and continues to escort her. If another male approaches, the paired male defends his mate by spreading his arms and displaying dark tiger color pattern toward the opponent. The mate-guarding male also approaches his opponent while beating his fins rapidly, and may also ink at the intruder. If the rival male does not retreat, the guarding male may dart at him, seizes him with the arms, and tries to bite him on the mantle. However, fights that escalate to biting are quite rare. Guarding males typically succeed in driving away offenders, except in cases when the offenders are much larger.

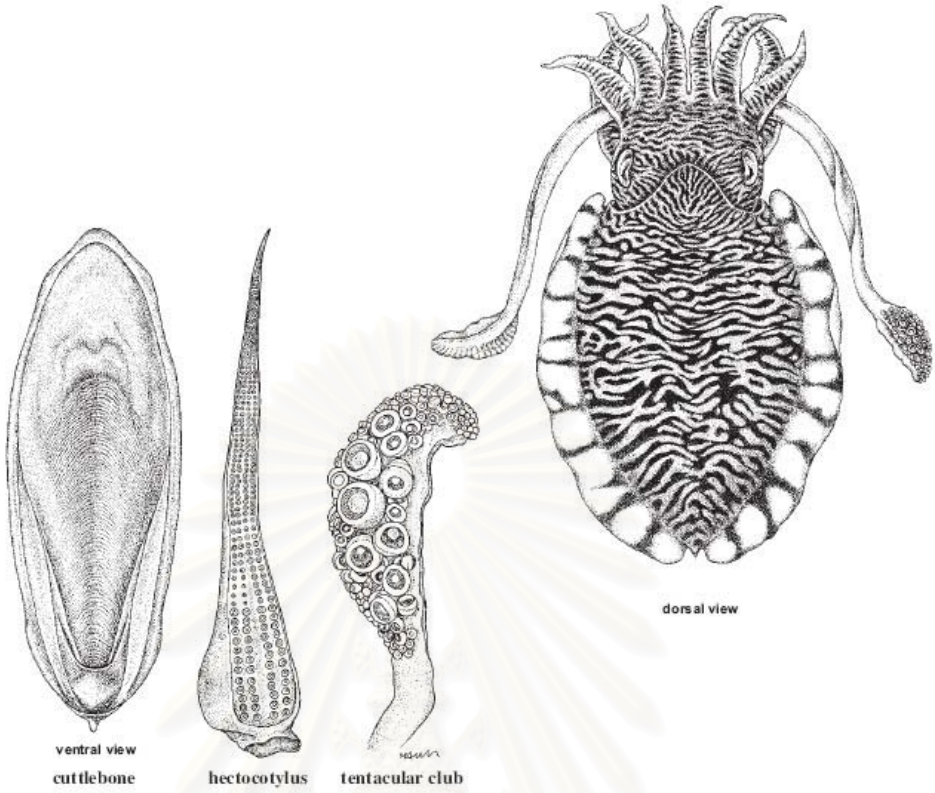


Fig. 1.3 Characteristics of the pharaoh cuttlefish, *S. pharaonis*. (modified from Jereb & Roper, 2005)

With regards to sperm competition, there has been no study conducted in *S. pharaonis* to demonstrate the presence of sperm removal behavior, last-male sperm precedence, and re-copulation behavior. The number of spermatophore transfers, and the number of spermatangia deposited during copulation have also never been analyzed quantitatively in this species.

1.4 Objectives of the current study

1) To investigate the presence of behavioral adaptations to sperm competition in *S. pharaonis*, namely sperm removal behavior, post-copulatory mate guarding behavior, and re-copulation behavior.

2) To investigate the presence of last-male sperm precedence in *S. pharaonis*, which if exists, would provide a supporting evidence for the effectiveness of sperm removal behavior, and provide an adaptive explanation for post-copulatory mate guarding.

3) To elucidate previously unknown details of copulation and mating behaviors in *S. pharaonis* (e.g. the number of spermatophore transfers per copulation, the total number of spermatangia deposited, the details of different visual displays, etc.), and to analyze them in the context of sperm competition when possible.

4) To compare the details of mating behaviors in *S. pharaonis* with what have been found in other species of cuttlefish.

CHAPTER II

MATERIALS AND METHODS

The study consisted of 3 experiments. Experiment I was done in an attempt to document details of copulation and to verify the presence of sperm removal behavior in *S. pharaonis*. Experiment II was done to investigate a possible consequence of sperm removal which is last-male sperm precedence. Lastly, experiment III was conducted to examine the male's other behavioral tactics to sperm competition, such as re-copulation, and post-copulatory mate guarding. All experiments were conducted at Rayong Coastal Fisheries Research and Development Center (Rayong CFRDC), Rayong Province, Thailand.

2.1 Experiment I

2.1.1 Animals and husbandry conditions

The experiment was conducted between 20 January and 23 February 2007. Adult *Sepia pharaonis* (n=30) were captured live using basket traps, which were placed in shallow coastal water off the coast of Rayong province, in the eastern region of the Gulf of Thailand. Once transported to the research center, the animals were kept in circular cement tanks of approximately 2-m in diameter filled to about 60 cm with seawater (Fig. 2.1a). The water in the tank was constantly replenished with natural seawater at the rate of about 1 $L.min^{-1}$. Water temperature, salinity, pH, and dissolved oxygen were maintained at about 28-32 C°, 25-35 psu, 7.0-8.5, and 5mg/L respectively, according to Nabhitabhata (1995).

The tanks were kept under natural dark-light cycles. Light intensity was reduced with camouflaging net to prevent algal growth (Fig. 2.1b). During mating trials which

were typically conducted between 6 pm – 2 am, artificial fluorescent lights were provided (Fig. 2.1c).

Fig. 2.1 The cement tanks that were used to keep the animals. a) Water was constantly supplied through the pipe at the edge and constantly drained through the pipe in the middle. b) Natural sunlight was filtered through camouflage net during day time. c) Fluorescent light was provided at night during the experiment.

Each animal was fed a whole dead fish (*Caranx leptolepis*, size=10-15 cm) twice a day at 8 am and 5 pm. Males and females were kept separated in different tanks. Separate rearing of each individual was usually a preferred condition. However, as the numbers of available tanks was limited (n=10), sometimes more than one individuals of the same sex were kept in the same tank (up to 3 males per tank, and up to 6 females per tank). Discrimination of sex was made using the stripe patterns (only the males had strong tiger stripes on their arms, Fig. 2.2b), the color patterns (only the males had a

patch of skin underneath their eyes (the cheek area), which when illuminated with a flashlight, showed green/orange iridescence, Fig. 2.2b), and the behavioral patterns (only the males exhibited the intense zebra display toward one another, Fig. 2.2a).

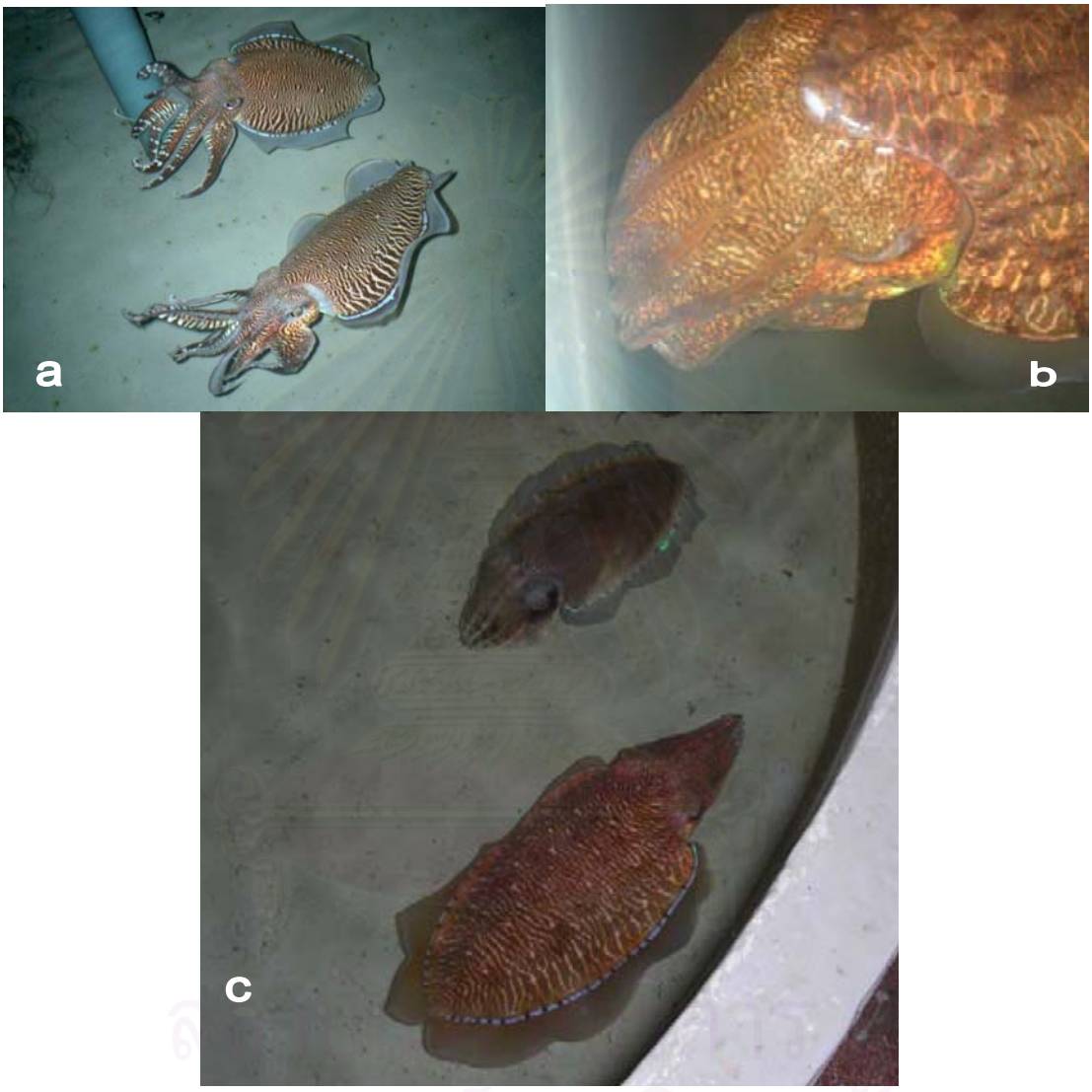


Fig. 2.2 Sexually dimorphic characteristics in *S. pharaonis*. a) Two males showing the Intense Zebra Display toward each other. b) The tiger pattern on the mantle and the arms of the male. c) A male (below) and a female (above).

2.1.2 Observation and analysis of mating behavior

To investigate the details of copulation behavior in *S. pharaonis*, and to determine whether or not the male of this species exhibits sperm removal behavior, each female (n=18) was arranged to mate twice consecutively with two different males (n=8 in different pair-combinations, Table 2.1). Matings were conducted and observed in a 0.6 x 1.5 x 0.8 m^3 rectangular glass tank (Fig. 2.3a-b). Sea water from the cement tanks that the animals were kept in was used to fill about 3/4 of the glass tank before each of the mating trials. Immediately after copulation began, a 400W spot light (Fig 2.3d) was turned on to lit the tank from beneath. The behaviors of the copulating couple were observed and filmed from underneath. A hand-held digital video camera was used for recording the behaviors. During copulation, the camera's ventral field of view was focused primarily on the male's arms, the male's funnel, and the female's buccal area (Fig 2.3c).

To determine the duration of the copulation, the duration of its different behavioral phases, and the number of spermatophore transfers that took place, the video recordings were played back and analyzed on a computer system, using the digital video editing software, Adobe Premiere Pro 1.0.

Prior to the mating trial, each female was anaesthetized by submerging in 1-2% ethanol solution for 1-2 min and checked for the presence of existing spermatangia from previous matings that she might have had. If any spermatangia were found attached to her buccal membrane at this stage (Fig 2.4b), they were removed by using a forceps.

Table 2.1 Mating scheme of experiment I. Male pairs that are highlighted with the same color indicate a reverse in order of mating between the same two males.

Trial #	Date (2007)	Male ID : First-Second	Female ID
1	Feb 1	Ma1-Ma2	Fem4
2	Feb 3	Ma3-Ma1	Fem6
3	Feb 4	Ma1-Ma3	Fem7
4	Feb 10	Ma2-Ma1	Fem8
5	Feb 11	Ma4-Ma3	Fem9
6		Ma5-Ma1	Fem10
7	Feb 12	Ma3-Ma4	Fem11
8		Ma1-Ma5	Fem12
9	Feb 13	Ma4-Ma2	Fem13
10	Feb 18	Ma8-Ma9	Fem15
11	Feb 19	Ma9-Ma8	Fem16
12		Ma10-Ma1	Fem17
13	Feb 20	Ma2-Ma4	Fem18
14		Ma1-Ma10	Fem19
15	Feb 21	Ma9-Ma10	Fem20
16		Ma2-Ma1	Fem21
17	Feb 22	Ma2-Ma9	Fem22
18		Ma10-Ma1	Fem14

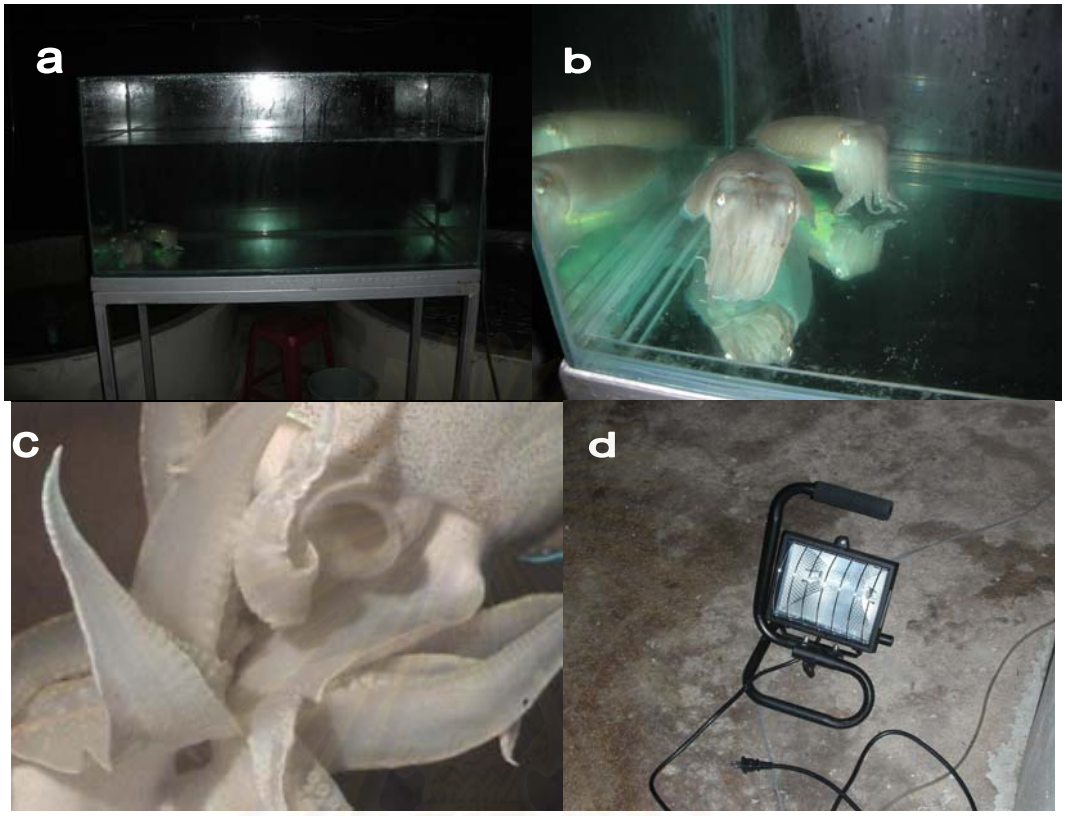


Fig. 2.3 a) and b) The experimental tank with transparent bottom, through which mating could be observed from underneath. c) The camera's ventral field of view captured movements of the arms and the funnel during mating. d) A 400W spot light was used to lit the tank from below during mating.

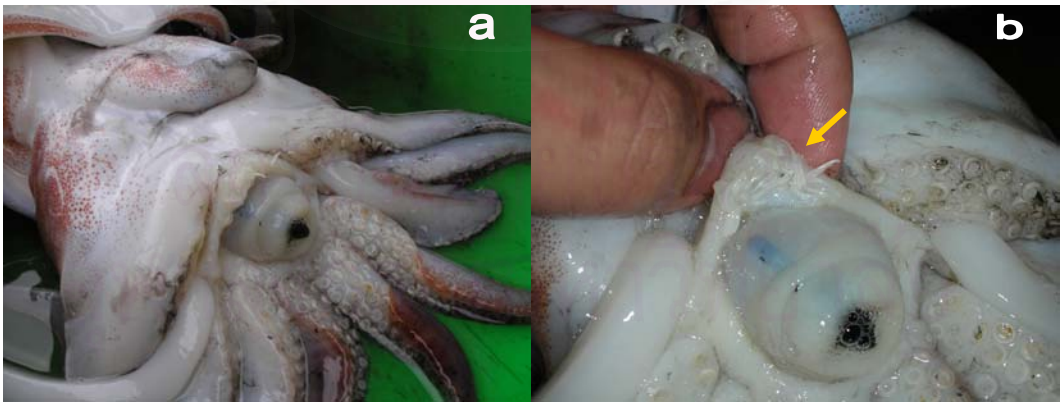


Fig. 2.4 a) Ventral view of a female's mouth area. b) Close up of the female's ventral buccal membrane, the site that the male usually attaches spermatangia to during mating. Numerous attached spermatangia can be seen in this picture (arrow).

Each experimental trial began by capturing a female cuttlefish gently using a net, and transporting her to the observational glass tank. A male was then captured and released into the same tank by the same method. The experimenter sat underneath the tank and prepared to film the mating as soon as it occurred. Most of the times, copulation began rather quickly (within 5-10 min after the release of the male), although in some trials, it may have taken much longer due to the male showing no sexual interest, or the female showing strong rejection toward the male. If 30-60 min had passed without copulation, one or both of the individuals may need to be replaced by others, or the trial may be delayed until the next day.

Fig. 2.5 a) Anaesthetized female in 1-2% ethanol. b) and c) Application of dye to the attached spermatangia using a paint brush. d) The spermatangia attached by the second male (white) and those that remained from the first mating (purple).

Immediately after the first copulation was over, both the male and the female were carefully taken out of the experimental tank. The male was returned to the cement

tank where it was originally kept. To color-label the spermatangia deposited during the first mating, the female was anaesthetized by putting it into a small container (vol = 4 L) filled with 1-2% ethanol solution (Fig. 2.5a). Typically, after 1-2 minutes, the female's respiration rate began to slow down considerably, judging by the low frequency of closing and opening of the siphon. At this point, the female could tolerate a direct handling and would not jet violently or eject ink at the experimenter, although the arms could still move and put up a resistance. The female's ventral buccal membrane was carefully exposed using a rod or a forceps. Once the spermatangia attached to the inside of the membrane were clearly seen, a paint brush was used to apply a solution of vital dye on the spermatangia (Fig. 2.5b). Many applications were typically needed to ensure that all spermatangia had been thoroughly covered with paint (Fig. 2.5c). The dye was a half-half mixture of 0.1% Touludine Blue and 0.1% Rhodamine B, which had been proven during the preliminary trials to be the appropriate dye for this purpose.

The application of the dye usually took no longer than 1-2 minute. After it was finished, the female was quickly transferred to a floating basket in the nearest cement tank. She was left there until the influence of the anesthetic solution wore out (10-15 minutes). During this period, materials that fell to the floor of the experimental tank during the mating were identified and counted. These included intact spermatphores, empty spermatophore sheaths, and unsuccessfully placed spermatangia. After the count was finished, all the materials were completely siphoned out of the tank using a plastic tube, and the water was refilled to its original level.

After the female regained control over its body, and started swimming normally, she was transferred from the floating basket back to the glass tank to receive the second copulation. The second male was put in shortly after the female. Once, the second copulation began, it was observed and filmed from directly underneath in the same manner as the first one. The elapsed time between the end of the first copulation and the onset of the second copulation was recorded.

During the second copulation, an observation of purple-dyed spermatangia falling out of the female's buccal area would confirm that the second male did indeed remove spermatangia deposited by the first male. When the copulation was over, the male and the female were carefully taken out of the tank. The colored spermatangia that had been removed and now laid at the bottom were identified and counted (Fig 2.6b), along with other materials (Fig 2.6a), including empty spermatophore sheaths, intact spermatophores, and white spermatangia unsuccessfully placed by the second male.

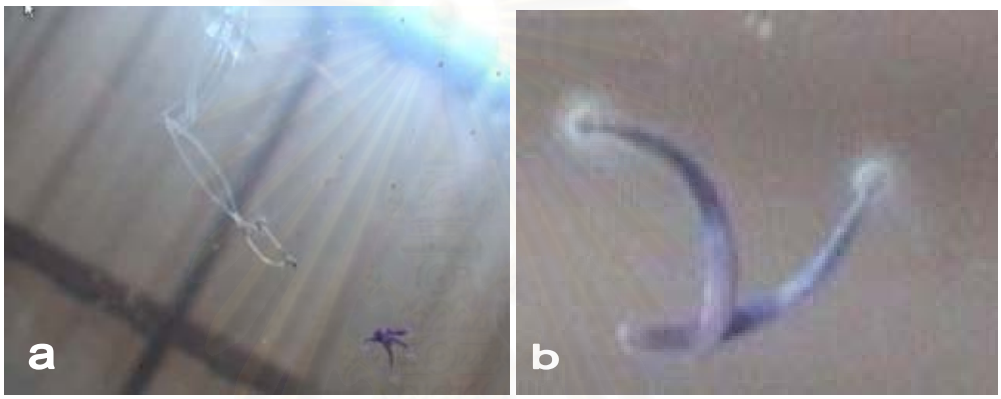


Fig. 2.6 a) Different kinds of material that fell to the floor of the tank during mating. The transparent spermatophore sheaths (upper most), the spermatangia that the second male failed to attach to the female (center), and the colored spermatangia from the first mating (bottom right). b) Close up of colored spermatangia.

The second male, once taken out of the experimental tank, was transferred back to its own living quarter. The female was put in 1-2% ethanol solution right after the second mating, and was left in there for about 2-3 minutes. Afterwards, the ventral buccal membrane of the female was cut out carefully to include every single spermatangia attached on it (Fig 2.7a). The obtained sample was put in a Petri-dish and later examined. In most cases, the ventral part of the buccal membrane was all that was needed from a female to count the number of spermatangia attached by each male. However, sometimes the male also attached spermatangia on other places such as the arms, hence in those cases, additional tissue samplings were required accordingly. The

females, in most of the trials, were killed in the process of tissue sampling (some survived and were killed later at the end of the experiment). Their bodies were measured for the mantle length (ML, Fig. 1.1a), before they were discarded.

Counting of spermatangia attached on the buccal membrane sample was done under a stereo microscope. Each spermatangium that had already been counted was pulled out one by one using a forceps, until none was left on the membrane (Fig 2.7c-d). Purple-dyed spermatangia were classified as the spermatangia that came from the first mating, whereas the white ones were classified as the spermatangia deposited by the second male (Fig. 2.7b).

Over the course of the experiment, some males died naturally, while the rest were killed after all the mating trials were finished. Their bodies were measured for mantle lengths. The male's spermatophore storage sac called the Needham's organ was dissected and measured for its length, as well as the number of spermatophores inside.

The experiment consisted of 18 trials. Eighteen different females were used, one for each trial. The same female was never used twice, whereas the males (n=8) were paired together in 9 different combinations, which sometimes included the same individual e.g. Ma1-Ma3 and Ma1-Ma2. Seven of these combinations also had at least one repetition done in reversed order. For example, there was a trial in which Ma1 mated first and Ma2 mated second, and then there was another reversal trial in which Ma1 mated second and Ma2 mated first. Mating trials that involved the same male were conducted at least 1 day apart and at most 9 days apart. Table 2.1 summarizes the dates of the trials, and the identities of individuals used in each of them.

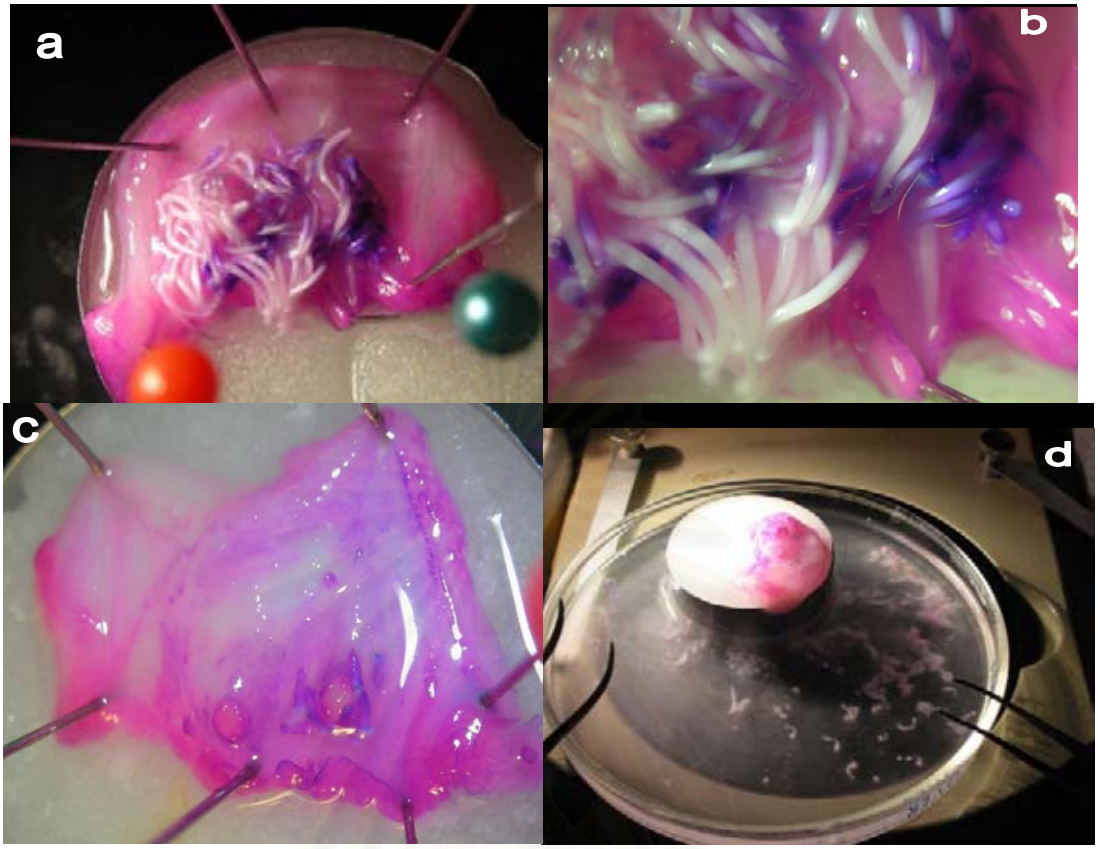


Fig. 2.7 a) The female's ventral buccal membrane was cut out, pinned, and examined under the microscope. b) Spermatangia deposited from the first mating (purple) and from the second mating (white). c) and d) A forceps was used to pull spermatangia out one by one until none was left on the membrane.

2.1.3 Quantitative and statistical analysis

For first matings, the total number of spermatophores that the male put out during copulation was calculated by adding the number of removed purple spermatangia found on the floor after the second mating, the number of purple spermatangia that remained on the female's buccal membrane after the second mating, the number of purple spermatangia that were misplaced on other parts (e.g. arms) of the female, and the number of spermatangia and intact spermatophores that fell to the bottom of the tank during the first mating. The number of spermatangia originally deposited by the first male prior to second male's removal was calculated by adding the

number of purple spermatangia found on the female's buccal membrane after the second mating to the number of removed purple spermatangia found on the floor after the second mating.

For second matings, the total number of spermatophores that the male put out during copulation was calculated by adding the number of white spermatangia found attached to the female's buccal membrane after the second mating, the number of white spermatangia that were misplaced on other parts (e.g. arms) of the female, and the number of white spermatangia and intact spermatophores that fell to the bottom of the tank during the second mating.

The average number of spermatophores that were passed out during each transfer was calculated by dividing the total number of spermatophores that were put out during the entire copulation with the number of transfers that occurred in that copulation.

Statistically significant differences between the first and the second matings on the following factors were determined using *t* test: (1) the duration of the removal phase of copulation, (2) the duration of the placement phase of copulation, (3) the average number of transfers per copulation, (4) the total number of spermatophores that were put out per copulation, (5) the number of spermatophores that were passed during each transfer, and (6) the overall mating duration.

Pearson correlations were obtained to determine the relationships between the following pairs of potentially related factors; (1) the size of Needham's organ and body size of the male, (2) the number of spermatophores remaining in Needham's organ after death and the size of Needham's organ, (3) the number of spermatophores remaining in Needham's organ after death and body size of the male, (4) body size of the male and the number of spermatophores that were passed during each transfer, (5) the size of Needham's organ and the number of spermatophores that were passed during each

transfer, (6) the overall mating duration and the duration of the placement phase, (7) the number of transfers per copulation and the duration of the placement phase, (8) the size of Needham's organ and the duration of the placement phase, (9) body size of the male and the duration of the placement phase, (10) the size of Needham's organ and the number of transfers per copulation, (11) body size of the male and the number of transfers per copulation, (12) the size of Needham's organ and the total number of spermatophores that were put out per copulation, (13) body size of the male and the total number of spermatophores that were put out per copulation, (14) the size of Needham's organ and the overall mating duration, (15) body size of the male and the overall mating duration, (16) the number of spermatangia removed and the number of spermatangia deposited during the first mating, (17) the percent of spermatangia removed and the number of spermatangia deposited during the first mating, (18) the duration of the removal phase and the number of spermatangia removed, (19) the duration of the removal phase and the percent of spermatangia removed, (20) the duration of the removal phase and the overall mating duration, (21) the duration of the removal phase and the duration of placement phase, (22) the duration of the removal phase and the number of spermatangia deposited during the first mating, (23) the duration of the interval between the two copulations and the duration of the removal phase, (24) the duration of the interval between the two copulations and the number of spermatangia removed, (25) the duration of the interval between the two copulations and the percent of spermatangia removed, (26) the size of Needham's organ and the number of spermatangia removed, (27) the size of Needham's organ and the percent of spermatangia removed, (28) body size of the male and the number of spermatangia removed, (29) body size of the male and the percent of spermatangia removed, (30) the total number of spermatophores that were put out by the remover and the number of spermatangia removed, (31) the total number of spermatophores that were put out by the remover and the percent of spermatangia removed, (32) the overall mating duration and the number of spermatangia removed, (33) the overall mating duration and the percent of spermatangia removed, (34) the duration of the first male's placement phase

and the number of spermatangia removed, and (35) the duration of the first male's placement phase and the percent of spermatangia removed.

2.2 Experiment II

To assess the pattern of sperm precedence in *S. pharaonis*, each female was arranged to mate with 2 different males consecutively. The resulting offspring were collected, and were to be tested for paternities in order to determine the relationship between the father's mating order and the proportion of offspring which it sired. The DNA samples of both the parents and the offspring had already been collected, and the primers used for DNA fingerprinting had already been screened and chosen from a pool of randomly generated markers. However, after a few attempts at the analysis of paternities, the preliminary results based on the chosen genetic markers were considered inconclusive and unreliable. Therefore the rest of the test was canceled, and the paternity data were not obtained. The following sections describe the methodology of the parts that had been done before the experiment was terminated.

2.2.1 Animals and husbandry conditions

Adult *S. pharaonis* (15 males, mean ML=20 cm; 14 females, mean ML=21 cm) were obtained by the same mean and kept in the same conditions as already described in experiment I. Only minor details differed in that when many animals were kept in the same tank, a divider made of wooden frame and plastic net was provided to prevent fighting between the males, and to ensure that the eggs laid in each compartment belonged to the female living in it (Fig. 2.8). Also, to deplete the sperm stock that may have already been stored from the wild, each newly captured female was allowed time to lay at least 2 clutches of eggs before it was used in the mating trial.

Fig. 2.8 Dividers made of wooden frames and plastic nettings were used to separate individuals living in the same tank.

2.2.2 Mating trials and the resulting eggs

Mating trials (n=14) and egg collections were conducted during 2 periods; January 29 to March 13, 2006, and June 17 to September 2, 2006. At the start of each trial, the male was transported to the cement tank in which the female was kept. Then, they were allowed to interact until mating ensued. After the copulation was over, the male was immediately separated and returned to its own living quarter. The second male was put in the female's tank afterward. He was allowed to copulate once, and then returned to his living quarter. The durations of both matings, and the time elapsed between them were recorded using a stopwatch. Table 2.2 summarizes identities of the males and the female used in each trial, and the date in which it was conducted.

Table 2.2 Mating scheme of experiment II.

Trial#	Male ID, first/second	Female ID	Date of first mating (2006)
1	MA / MD	Fe5	08/02
2	MD / MA	Fe4	09/02
3	MB / MC	Fe3	15/02
4	MC / MB	Fe6	16/02
5	M1 / M4	F1	25/06
6	Br / M3	F7	25/06
7	Br / M1	F3	14/07
8	M7 / M14	F13	02/08
9	M5 / M7	F8	02/08
10	M13 / M11	F11	03/08
11	M6 / M9	F18	03/08
12	M14 / M7	F15	07/08
13	M9 / M6	F17	07/08
14	M11 / M9	F16	07/08

After receiving two copulations, the female was left in her own tank to lay eggs. A 30 cm x 45 cm fishing net tied to a brick, or a four sided mesh made out of wooden sticks was provided as the laying material (Fig. 2.9a-b). Three to four days old eggs were counted, and separated from the mesh by hands (Fig. 2.9c), then transferred to a floating basket with an appropriate label (Fig. 2.9d), and were kept there until hatching. After the matings, each female was expected to lay at least 2 successive clutches of eggs. Each clutch was noted for the date in which it was laid, the number of eggs that it had, the number of eggs that developed to hatching, and the date of hatching.

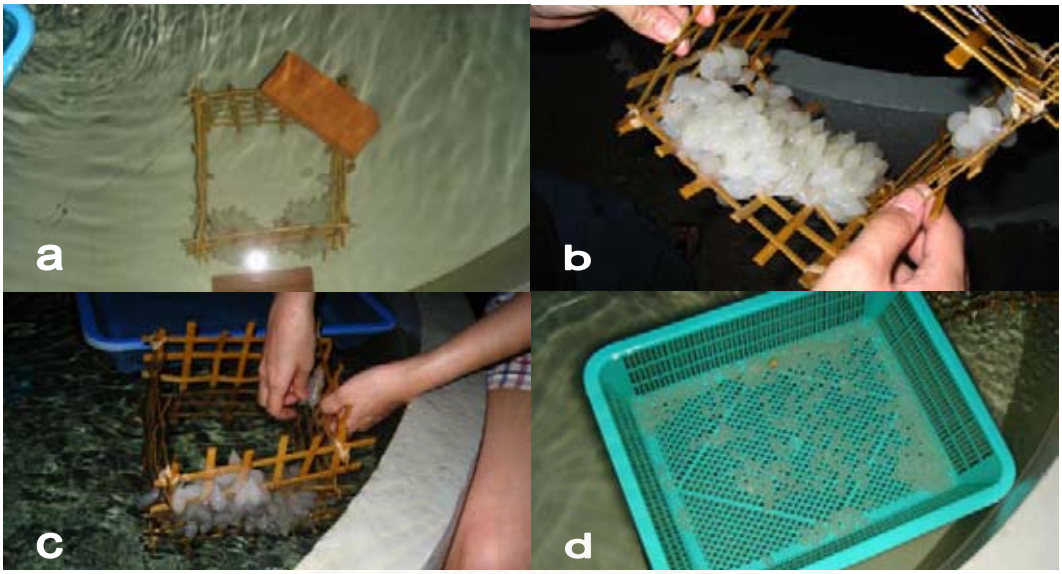


Fig. 2.9 a) and b) The egg mass attached to the wooden mesh. c) and d) Several days old eggs were ripped from the mesh one by one and were put in a floating basket during incubation period.

2.2.3 Tissue sampling and DNA extraction

For adult individuals, tissue sampling was conducted by withholding the animal in a net, and quickly cutting off a small piece of tissue (5 mm x 1 cm) from the tip of its tail fin using a scissor. The obtained samples were put in microfuge tubes (1.5 ml) containing absolute ethanol, and were transported back to the laboratory at Chulalongkorn University, Bangkok, Thailand, for DNA extraction and further analysis. Once arrived at the laboratory, the samples were kept at –70°C inside a freezer.

The young, at their final stage of development (ready to hatch, Fig 2.10a), were squeezed out of their egg shells directly into a bowl (Fig 2.10b), and then collected from there (typically, the clutch was moved into a bowl 2-3 days before the day of first hatching, and as soon as some hatchlings emerged, the rest of the developed eggs were squeezed out by hands). All hatchlings were collected from each clutch. Hatchlings collected were instantly fixed whole in absolute ethanol, and put in

appropriately labeled small plastic jars (Fig 2.10c), before transported back to the laboratory and kept at –70°C.

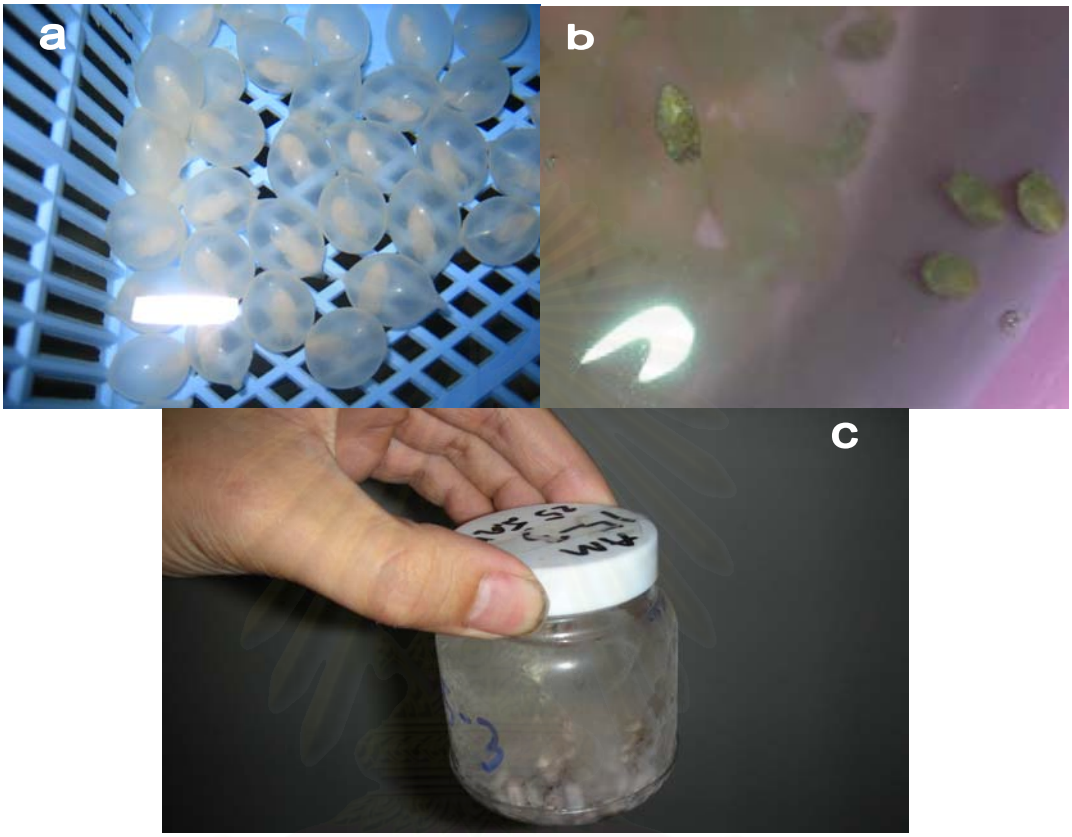


Fig. 2.10 a) Eggs ready to hatch. b) Hatchlings about 0.5-1 cm long. c) Hatchlings collected and put in a plastic jar filled with absolute ethanol.

For total DNA extraction, a small piece of tissue (about 1-4 mm^3) was dissected from each sample, and digested at 55°C for 3 hours, using 485 µl of TEN + 1% SDS buffer (50 mM Tris-base; pH 8.0, 100mM NaCl, 5 mM EDTA; pH 8.0, 1% SDS (w/v)) mixed with 15 µl of protinase K solution (10 mg/ml). After the digestion was complete, 400 µl (1 volume) of phenol/chloroform solution was added to each tube and shaken vigorously. The resulting milky solution was then spun at 10,000 rpm for 5 minutes. The centrifugation separated the aqueous phase, which contained dissolved DNA, from the organic phase, which contained digested proteins and other impurities. The aqueous phase (about 400 µl) was removed carefully and transferred to a fresh microfuge tube.

To precipitate the DNA, 800 µl (2 volumes) of absolute ethanol was added, and the mixture was incubated at –20°C overnight, or at –70°C for about 1 hour. The precipitated DNA was recovered by centrifugation at 14,000 rpm for 15 minutes. Ethanol was removed with care, and the DNA pallet at the bottom of the tube was left to dry at room temperature (typically overnight but sometime over 4-5 hours). The dried DNA was re-dissolved in 30 µl of TE buffer (10 mM Tris, 0.1 mM EDTA; pH 8.0), and stored at –70°C until further manipulation.

The extracted DNA was checked for its presence and its concentration by electrophoresis. One microlitre of DNA solution was mixed with 2 µl of loading dye (standard stain orange G, 40% glycerol) and 7 µl of distilled water, then loaded into 0.8% agarose mini-gel containing 4 µl of ethidium bromide solution (500 µg/ml). Electrophoresis was run at 80 V for approximately 30 minutes. The resulting band was visualized under UV light, and photographed using a gel documenting system. The fluorescent level of the band indicated the relative concentration of DNA in the sample. The sample's concentration was adjusted (dilution by adding distilled water) until yielding a band with the desired level of fluorescent, which suggested the final concentration of about 25µg/ml, suitable for use in PCR amplification (DNA solutions with known concentrations were used as reference).

2.2.4 ISSR-PCR and primer screening

Since there had been no prior knowledge regarding genetics of *S. pharaonis*, ISSR-PCR using random primers was selected as a technique for obtaining individuals' DNA fingerprints. 48 random ISSR primers (Appendix A) were screened, and only the ones that exhibited polymorphism between the parents, and generated clear and reproducible banding patterns were chosen for further use.

Twenty five microlires of amplification reactions contained 2.5 µl of 10X reaction buffer (20 mM Mg^{2+}), 2.5 µl of 2.5 mM dNTPs, 0.25 µl of 10 pmol primer, 0.2 µl of 5 unit

of *Taq* DNA polymerase, and 10-25 ng of genomic DNA. PCR cycles began with 94°C for 5 min, followed by 45 cycles of 94°C for 45 sec, 41°C for 45 sec, and 72°C for 2 min. The last cycle was followed by the final extension at 72°C for 10 min.

The resulting PCR products were mixed with 5 µl of loading dye (standard stain orange G, 40% glycerol), and were electrophorosed at 100 V for 3 hrs and 30 min, in a 2.0% agarose gel, containing 13 µl of 500 µg/ml ethidium bromide, and submerged in 0.5X TBE buffer (8.9 mM Tris-HCl, 8.89 mM boric acid and 2.5 mM EDTA; pH 8.3). The fluorescent banding patterns were visualized under UV light and photographed using the gel documenting system. Sizes of the identified bands were determined relative to a 100-bp DNA ladder.

2.2.5 Statistical analysis

For data on mating durations, the difference between the mean duration of the first and the second mating were analyzed statistically using *t* test. Pearson correlation was obtained to determine the relationship between the duration of the interval between two matings and the duration of the second mating.

Paired *t* test was used to determined statistical difference between the first clutch of eggs and the second clutch of eggs with regards to the number of eggs that they contained, and the hatching rates. Pearson correlations were also obtained to determine the relationship between (1) the number of eggs laid in the first clutch and the number of eggs laid in the second clutch, and (2) the hatching rate of the first clutch and the hatching rate of the second clutch.

2.3 Experiment III

2.3.1 Animals and husbandry conditions

The experiment was conducted between 19 August and 19 September 2005. Adult *S. pharaonis* (10 males, mean ML=20 cm ; 4 females, mean ML=20 cm) were obtained by the same mean and maintained in the same conditions as already described in experiment I. Although during this experiment, no more than one animal was ever kept in the same tank.

2.3.2 Testing re-copulation

Re-copulation with the same female could serve as a useful strategy to improve fertilization success during sperm competition. It may also serve as a counter measure to sperm removal. To verify the presence of this behavior in *S. pharaonis*, each male was put in the same tank with 2 females, and was allowed to mate twice. The male's choice of mate was recorded to determine whether or not he tended to re-copulate with the same female for the second time.

Twenty five mating trials were conducted in a cement tank at night (between 7 PM-2AM), and were filmed from above using a hand-held digital video camera (the same as in experiment I). Lighting was provided by 2 fluorescent lights, placed at the opposite edges of the tank (Fig. 2.11). Oxygenation and water circulation were stopped during the trial, so that they did not disturb the surface, and that the video taken would contain the clearest image possible. At the beginning of each trial, 2 females were put into the tank first, followed by the male. Filming started as soon as the male was released, and continued through the first mating, the second mating, and also the period in between. The duration of time spent before the first mating, the durations of both copulations, and the amount of time elapsed between them were recorded. If the

male spent more than 1-2 hours in the tank and still showed no sign of interest in mating, the trial would be terminated, and the result would be marked as “no mating”.

Fig. 2.11 Two fluorescent lights were positioned at the edge of the tank during the filming of each mating trial.

Twenty five trials consisted of 4 different pairings of the females were conducted. The pair that was used the most (14 times) was the one between female fB and fE. A pair of female underwent 1-4 consecutive trials per night. Each male was tested with the same pair of females 1-3 times. Consecutive mating trials, for each male, were conducted at least one day apart, except in 2 cases, in which the same male was put through 2 trials within the same day. Table 2.4 summarizes identities of the male and the females used in each trial, and the date it was conducted.

Table 2.4 Mating scheme of experiment III.

Male IDs	Female ID	No. of repeats	Dates conducted (2005)
mA	fA, fB	2	19/08, 19/08
mB	fA, fB	1	21/08
mC	fB, fC	1	28/08
mD	fB, fC	1	29/08
mD	fB, fE	3	29/08, 14/09, 18/09
mE	fD, fE	1	29/08
mF	fB, fE	3	13/09, 14/09, 18/09
mG	fB, fC	1	30/08
mG	fB, fE	3	13/09, 14/09, 18/09
mH	fB, fE	3	13/09, 14/09, 18/09
mI	fB, fC	2	29/08, 30/08
mJ	fB, fC	2	30/08, 18/09
mJ	fB, fE	2	14/09, 18/09

2.3.3 Observation of pre- and post-copulatory behaviors

Pre-copulatory period began when the male was release and lasted until the first copulation occurred. Behaviors of the animals during this period were observed and recorded, with an emphasis on the male's courtship behaviors, the female's accepting or rejecting behaviors, and the movements, the postures, the color/stripes patterns associated with them.

Post-copulatory behaviors were observed and recorded from the end of the first mating, to the onset of second mating. Emphasis was put on the male's mate guarding behavior during this period; how closely and how long he remained on guard, or in the

near proximity (within 1 body length) of the female whom he had recently mated, and whether or not he switched to pursue the other female whom he had not mated with.

2.3.4 Statistical analysis

Paired *t* test was used to determine the statistical difference between the durations of the first and the second copulation. Pearson correlations were obtained to determine the relationships between (1) the duration of the first copulation and the interval between the two copulations, (2) the duration of the first copulation and the duration of the second copulation, and (3) the interval between the two copulations and the duration of the second copulation.

CHAPTER III

RESULTS

3.1 Experiment I

3.1.1 The male reproductive anatomy

After death, examination of internal organs inside the mantle cavity revealed that every male (n=8) possessed a well developed reproductive-organ complex, located on the left side of the body, just underneath and posterior to the base of the left gill (when viewed ventrally, Fig. 3.1a). The male reproductive-organ complex consisted of a single testis, the highly coiled vas deferens, various accessory glands, and a large long spermatophore-storage sac called the Needham's organ (Fig. 3.1c). All of these structures were held together inside a translucent membrane, forming a complex that was separatable from the rest of the organ systems (Fig. 3.1b-c). The average length of the male's Needham's organ was 7.5 cm ($\pm$ 1.6 cm, range 5.5-10.5 cm, n = 8). The size of Needham's organ showed a significant correlation to the body size of the male (mantle length), thus a larger male tended to have a longer Needham's organ ($r = 0.76$, $p<0.05$, n=8, Fig. 3.2).

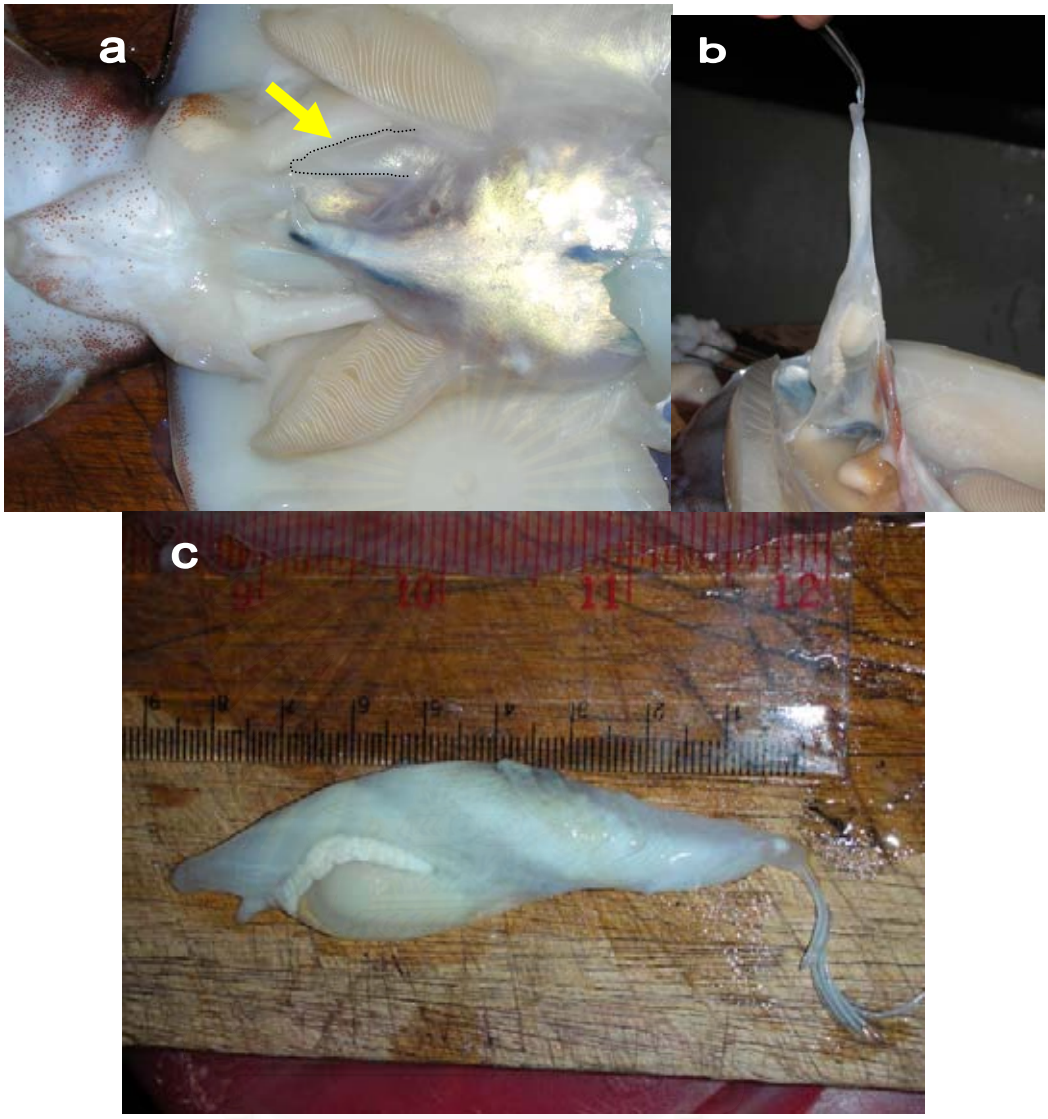


Fig. 3.1 Male reproductive anatomy. a) Ventral view of internal organs. Arrow is pointing at the distal portion of the Needham's organ, protruding from underneath the base of the left gill. b) After all the connective tissues have been cut, the whole reproductive-organ complex can be separated as a package. c) Close up of the reproductive-organ complex showing, the testis, the coiled vas deferens, the Needham's sac, and the spermatophores tightly packed inside.

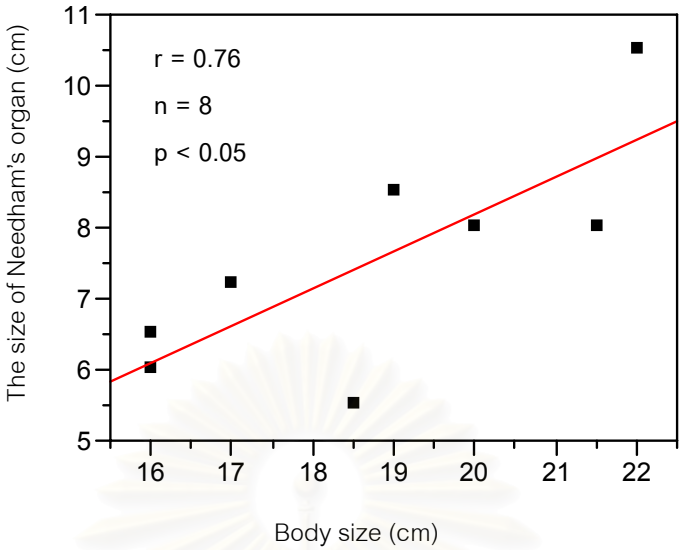


Fig. 3.2 Relationship between the body size of the male and the size of the Needham's organ.

The Needham's sac was typically full of tightly packed spermatophores (Fig. 3.3a). In 7 out of 8 males examined, almost all of these spermatophores were completely developed and were still intact (Fig. 3.6). In each male, only a few (5-10 spermatophores) were found still in various stages of development (some were spiral and bulged at the tip, others were much longer than usual, Fig. 3.3b-c). In one of the males (Ma5), almost all the spermatophores inside the Needham's sac had degenerated and appeared as white masses attached to the tips of empty sheaths (Fig. 3.3d). The particular male had been dead for many hours before it was dissected, and the degeneration may have happened during that period. Its spermatophores transferred while alive during the experimental trial were normal looking.

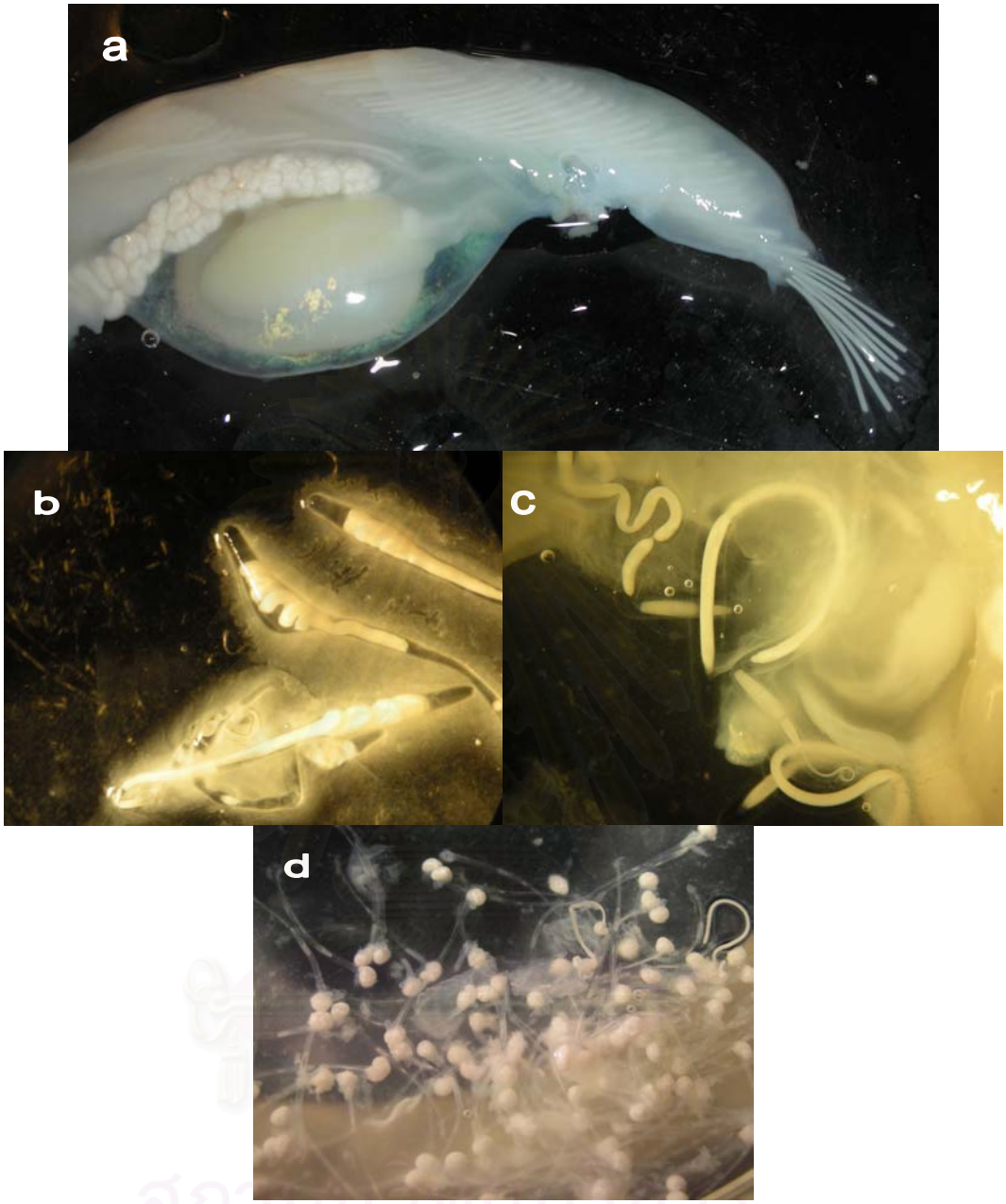


Fig. 3.3 a) Normal, fully developed spermatophores tightly packed inside the Needham's organ. Some are protruding from the opening at the tip. b) and c) Spermatophores that are still in various stages of development. d) Bursted, degenerated, abnormal spermatophores found in one male several hours after death.

An average of 193 (± 112, range 58-420, n = 8) intact and completely developed spermatophores were found inside each male's Needham's organ (for Ma5 the degenerated spermatophores were counted as if they were normal). There was no

significant correlation between the number of spermatophores and the size of the males, or the size of their Needham's organs.

Examination of external structures showed that, in every male, the mid-section of the left fourth arm was modified by a reduction in suckers (Fig. 3.4a). The modified section was termed the hectocotylized portion of the left fourth arm, or the hectocotylus. It is a specialized, sexually dimorphic structure that is used by the male during mating, for holding and transferring spermatophores to the female.

Fig. 3.4 a) A male's left fourth arm with reduced suckers, or the hectocotylized portion at the base (arrow). b) The right fourth arm of the same male. The hectocotylized portion is absent.

3.1.2 Spermatophore, spermatangia, and the mechanism of ejaculation

An intact spermatophore was approximately 1.5 – 2.0 cm long, and consisted of an outer case, an elongated white sperm mass, a cement body, a coiled ejaculatory organ, and a cap (Fig. 3.5 and 3.6). The cap was in turn connected to a thin thread which, when pulled, caused the cap to loosen and triggered an evagination of the spermatophore. The content, including the cement and the inner tube containing sperm mass, was slowly expelled out of the spermatophore during evagination (the whole process took about 10-15 sec, Fig. 3.7). Once the release was complete, the resulting white tube filled with spermatozoa could be separated from the empty sheath, and the structure was now called a spermatangium (Fig. 3.5c, and 3.8a-b). Each spermatangium was 0.5-0.8 cm long, and the male typically attached many of them on the female's buccal membrane during copulation (Fig. 3.8d, please note that it was the spermatangia that were attached, not the intact spermatophores).

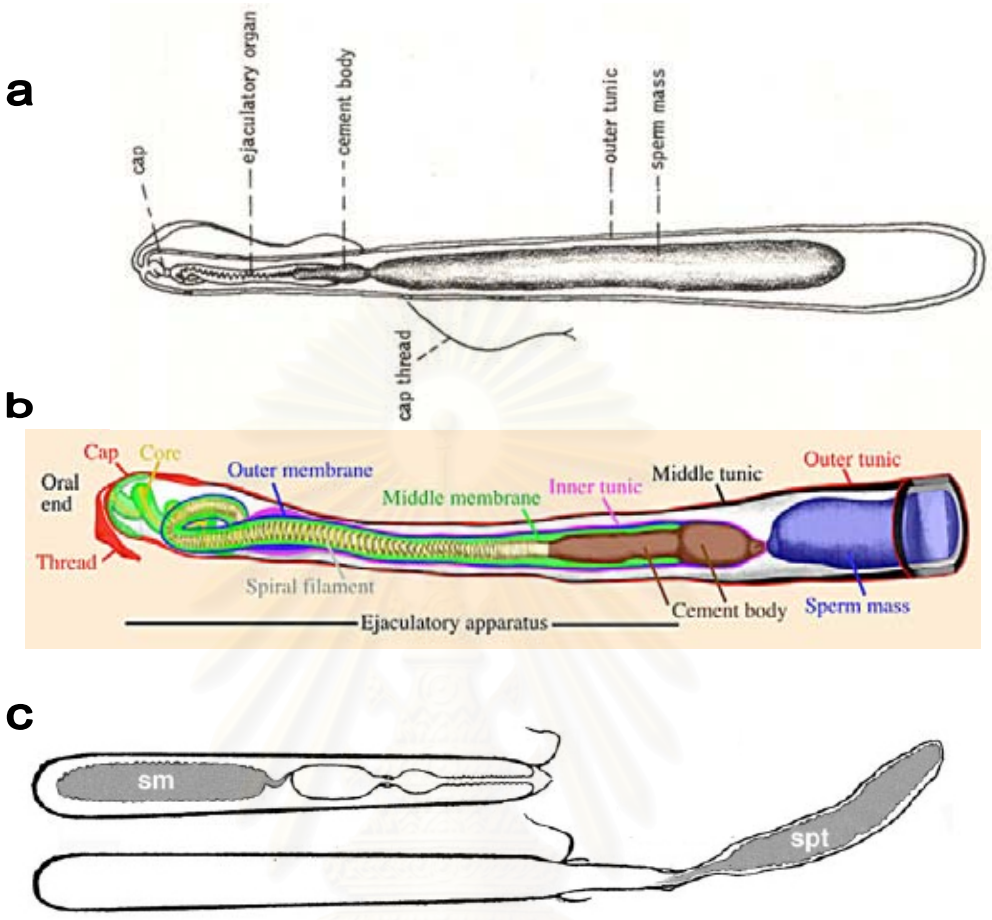


Fig. 3.5 a) Illustration of a typical cephalopod spermatophore showing different components (source: Tsai, 2006). b) Close up of the cap region showing the cement body, the coiled ejaculatory apparatus, the cap, and the thread (source: Young et al., 2000). c) An intact spermatophore (top) contains sperm mass (sm). When it is evaginated (bottom), the sperm mass becomes exposed and is re-termed a spermatangium (spt) (modified from Hanlon et al., 1999).

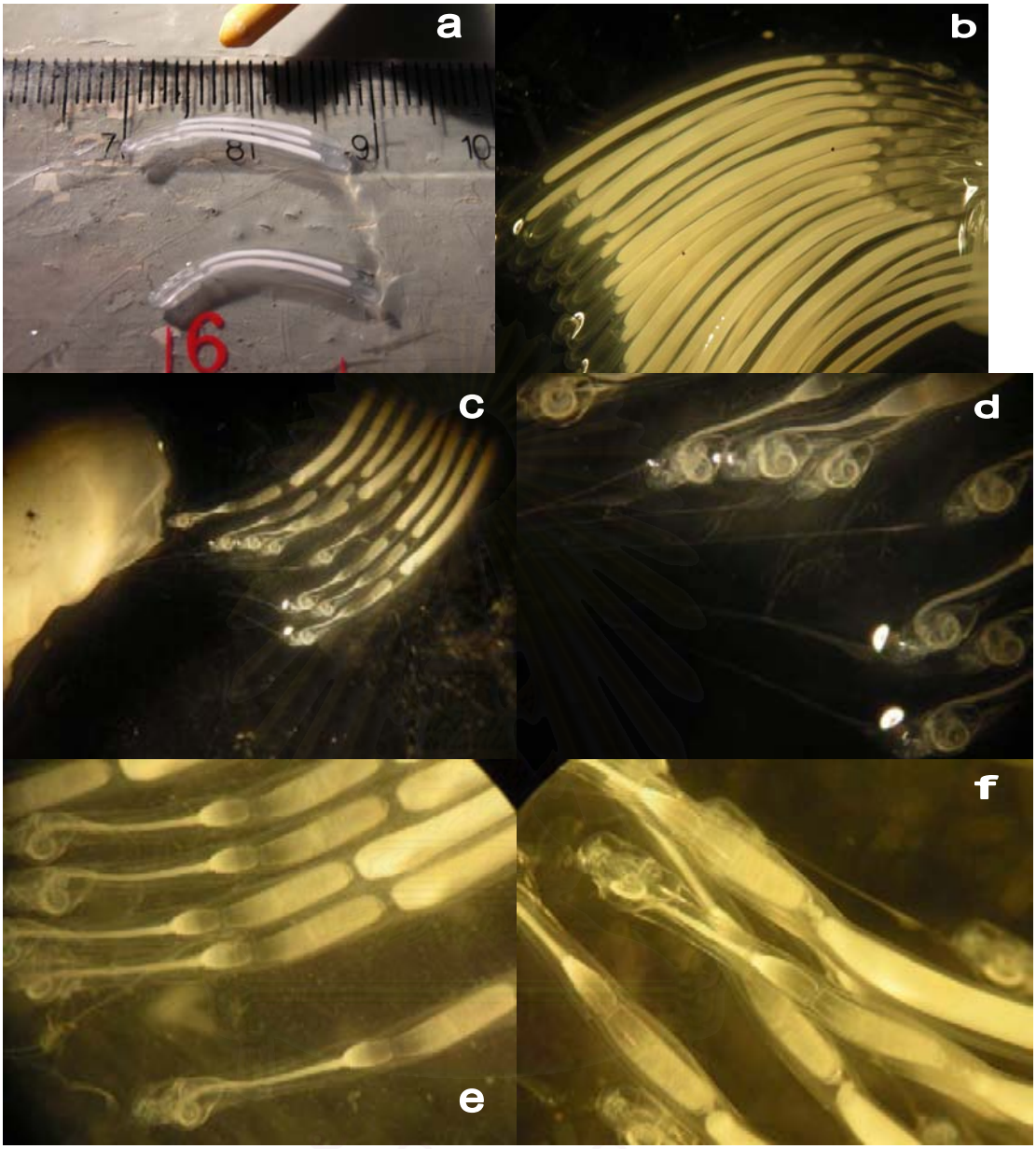


Fig. 3.6 a) and b) Intact spermatophores of *S. pharaonis*, 1.5-2 cm long. c) and d) Newly separated spermatophores have very thin threads connecting from the caps to the Needham's organ. e) and f) The base of the sperm mass, the cement body, the ejaculatory apparatus, and the cap.

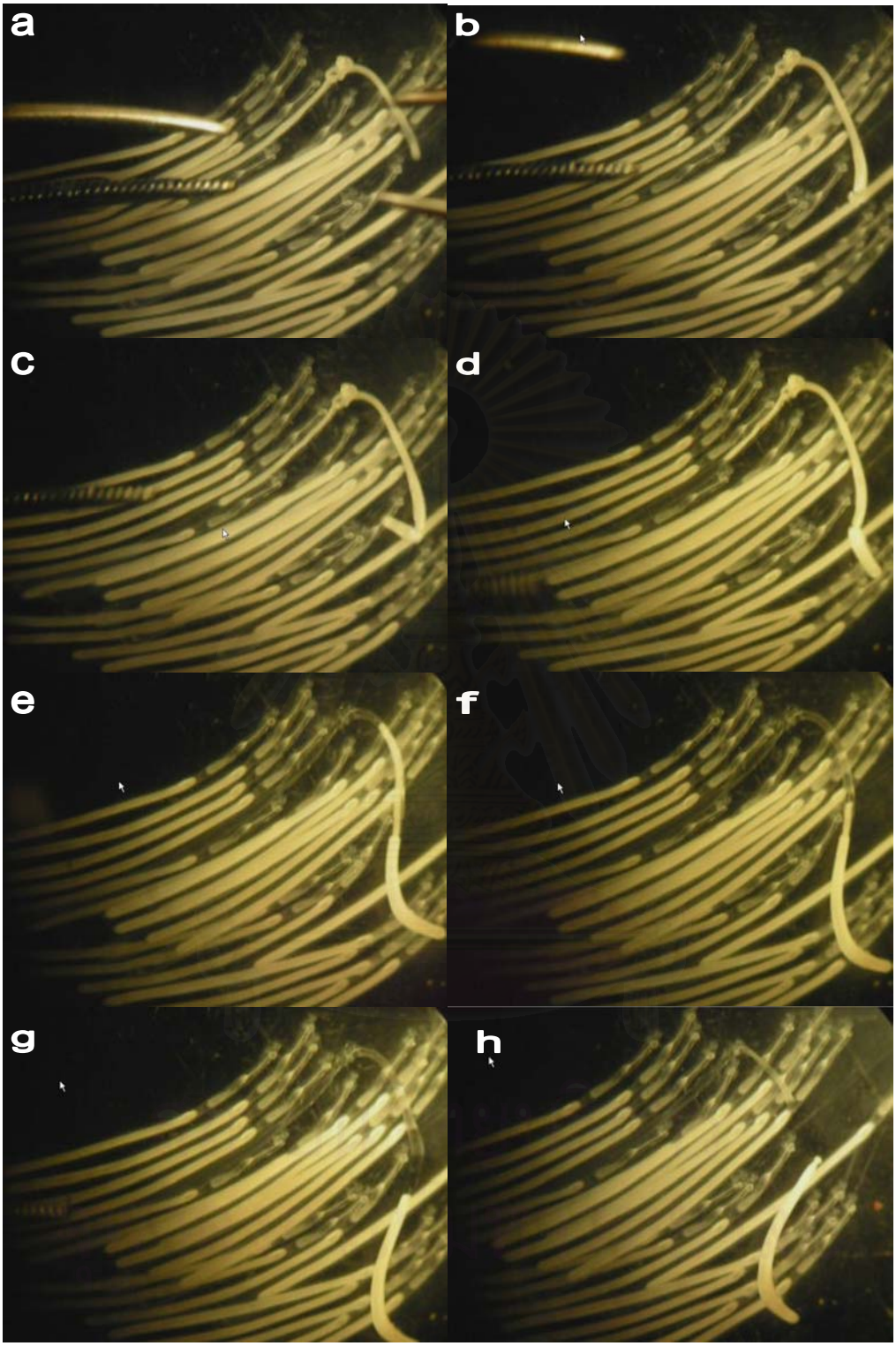


Fig. 3.7 a) to h) Chronological series of video captures showing the process of spermatophore evagination.

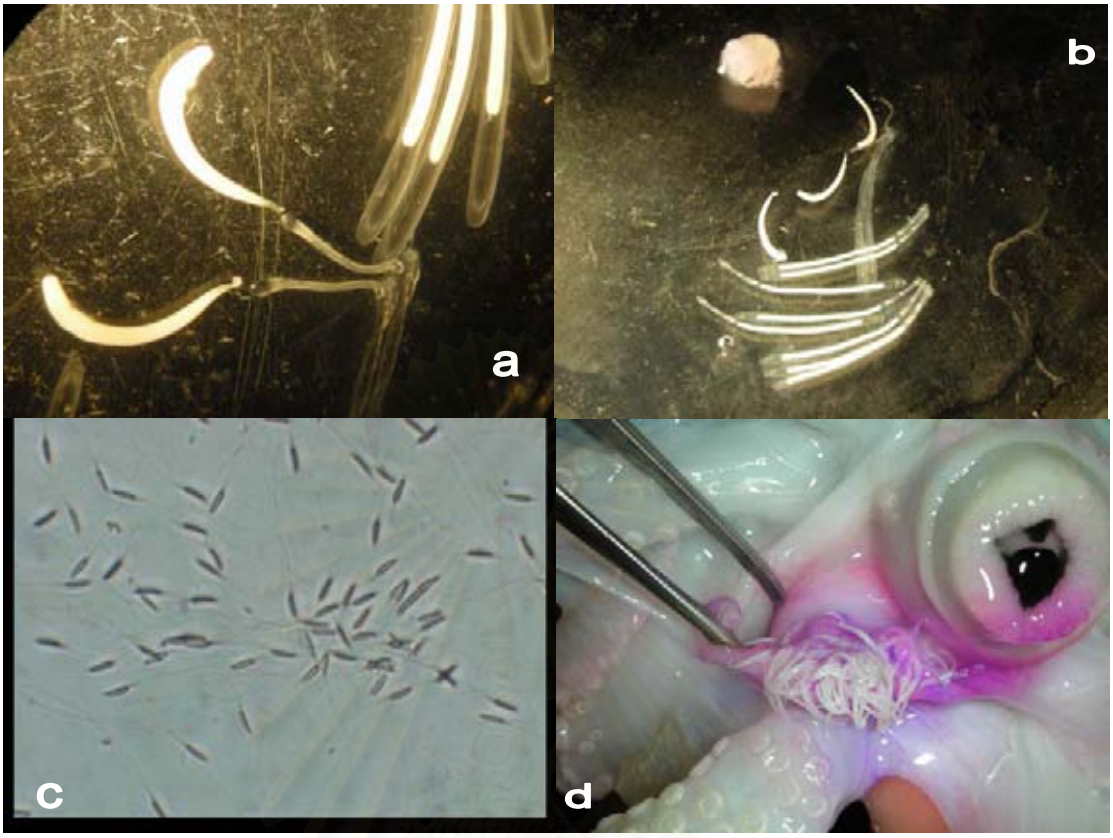


Fig. 3.8 a) and b) Newly emerged spermatangia (sickle shape). Some are still attached to the spermatophore sheaths. c) Sample of white mass inside spermatangia was found to contain numerous live sperm. d) Spermatangia that were found attached to the ventral region of the female's buccal membrane after mating.

3.1.3 Female buccal membrane and sperm receptacles

Each female (n=18) possessed a pair of seminal receptacles in the ventral buccal membrane (Fig. 3.9a, c). Each seminal receptacle contained a group of glandular sacs that shared a common duct, which opened to the exterior by a single pore on the inner surface of the ventral buccal membrane (Fig. 3.9d).

The paired receptacles function as a sperm-storage site. Spermatangia from the male are typically deposited on the inner surface of the female's ventral buccal membrane (Fig. 3.9b). To be stored inside the seminal receptacles, the sperm, presumeably, must travel from the spermatangia and enter the receptacles through the

pores on the lip of the buccal membrane. The actual mechanism that the sperm use to achieve this still remains unknown. However, the attached spermatangia themselves can also serve as sperm storage site for the female in addition to the receptacles. Nuad et al. (2005) reported that in *S. apama*, the female had access to sperm stored in both sites, and in fact, most eggs were fertilized by sperm originating from the spermatangia rather than from the receptacles.

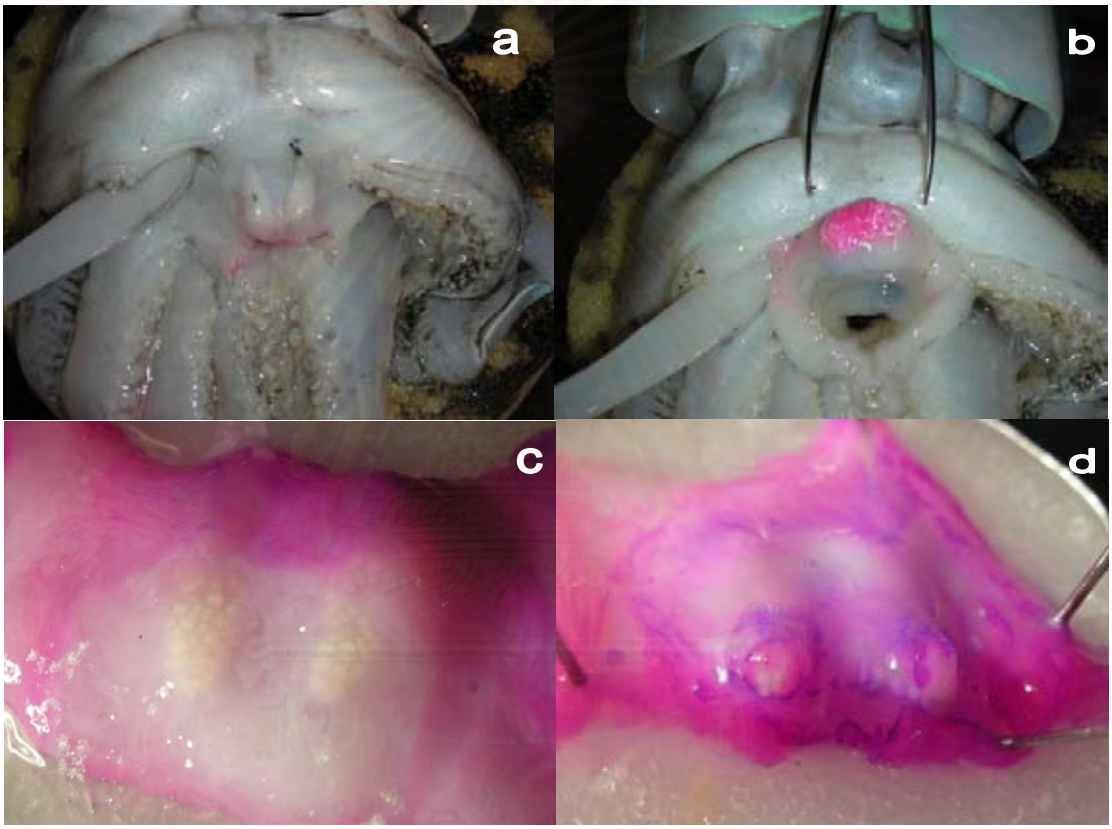


Fig. 3.9 a) Ventral view of a female showing the paired seminal receptacle inside the buccal membrane. b) The other side of the buccal membrane reveals attached spermatangia. c) Close up of the receptacles in a dissected buccal membrane showing clusters of glandular sacs that are used to store sperm. d) On the opposite side, there are 2 openings that lead to the receptacles.

3.1.4 Details of copulation

A total of 36 copulations were observed. The copulation sequence can be divided into three distinct phases: 1) the sperm removal phase, 2) the spermatophore transfer phase, and 3) the spermatangia placement phase.

3.1.4.1 The sperm removal phase

This phase occurred immediately after the mating pair had aligned themselves into the head-to-head position. If earlier, there had been spermatangia deposited by the previous male, the copulating male would begin removing them by using mainly its third pair of arms. The sucker sides of these arms were pressed against the ventral region of the female's buccal membrane. The male moved the arms slightly forward and backward in a grinding motion, which in effect, scraped off the attached spermatangia (Fig. 3.10). Typically, the removals of spermatangia were also accompanied by violent jerky pulling movements of the arms. The movement of the suction cups, and their teeth might have also played roles in cutting and clipping off the spermatangia, but their actions were not directly observed during this study.

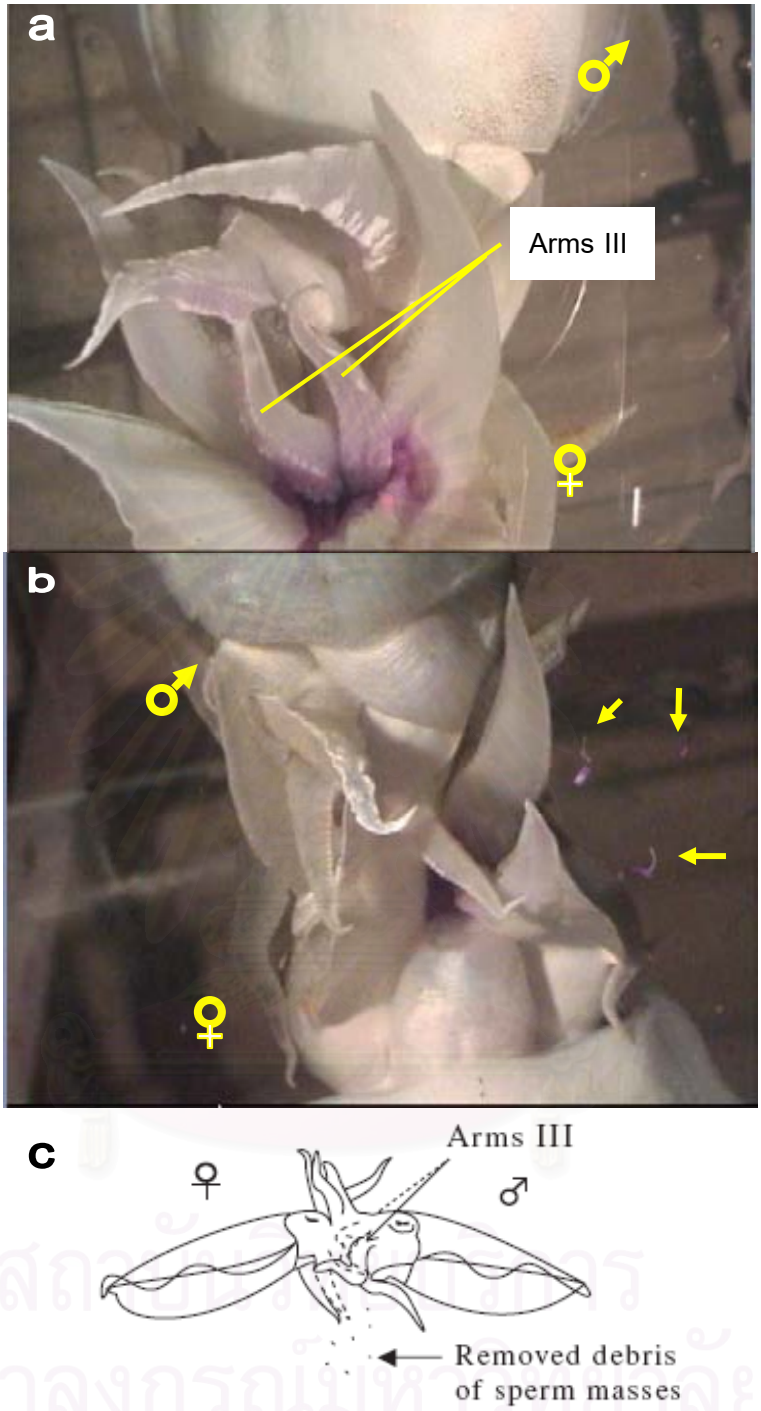


Fig. 3.10 Video captures of copulation from the ventral view during the sperm removal phase. a) The male used the third pair of arms to press and scrape against the female's buccal area. b) Arrows indicate the removed purple spermatangia falling to the floor. c) An illustration of sperm removal behavior from a lateral view (modified from Wada et al., 2005).

During this phase, the males pumped vigorously with their mantles and jetted large quantities of water through their funnels, but these pulses of flushing were not necessarily aimed at the female's buccal area directly. Also, removed spermatangia could be seen falling out even when the male was aiming his funnel at other directions. Both these observations suggested that flushing did not serve as the main mechanism for sperm removal. Its only apparent effect was blowing away pieces of spermatangia that had already been detached by the scraping and pulling actions of the arms.

The freshly removed spermatangia were purple in color, indicating that they came from the first mating (Fig. 3.11). They were removed either singly or sometimes in mass which consisted of several spermatangia tangled together. All removed spermatangia eventually sank and settled down at the bottom of the tank. The characteristic shape of spermatangia remained intact after they have been removed. Although their sizes varied depending on where they had broken off, all were still recognizable as spermatangia (Fig. 3.11).

The second male removed on average 22 spermatangia per mating ($\pm$ 15, range 0-47, n = 18). In terms of percentage, the second male removed on average 34.23% of the number of spermatangia previously deposited by the first male ($\pm$ 23.47%, range 0-92.86%, n = 18). In all except two trials, there were at least some spermatangia removed. However, in none of the trials did the second male completely remove all of the first male's spermatangia. Some spermatangia from the first mating always remained on the female buccal membrane afterwards (Fig. 2.7a-b).

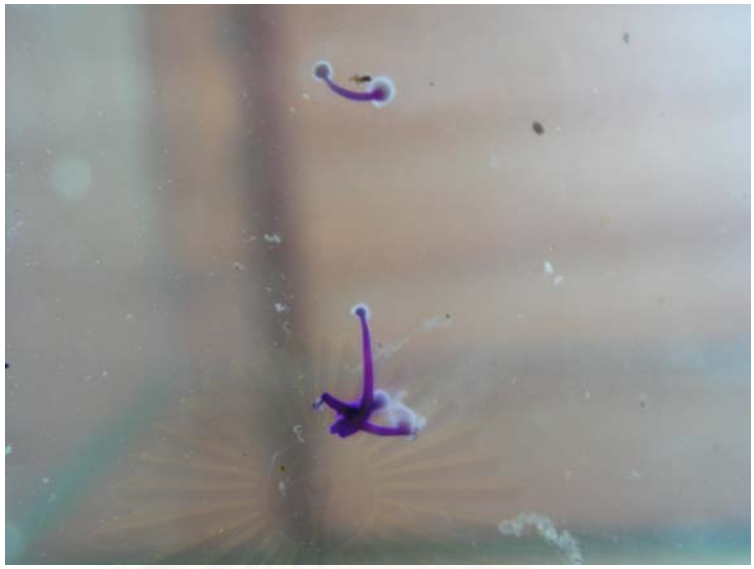


Fig. 3.11 Removed spermatangia contained the purple color.

The end of sperm removal phase was marked by the first occurrence of spermatophore transfer. After the male transfered his first spermatophore bundle to the female, no further removal of spermatangia from the previous mating was observed (except in one trial, during which, a mass of colored spermatangia was blown out after the male had already transferred his spermatophore twice.).

The second males spent on average 1 min 35 s in the removal phase ($\pm$ 1 min 5 s, range = 6 s – 4 min 20 s, n=18). The first males, before transferring their own sperm, also carried out the same kind of removal actions, even though there were no previously deposited spermatangia. The first male's equivalent of the removal phase lasted on average 2 min 45 s $\pm$ 1 min 9 s (range = 1 min – 4 min 46 s, n=18), which was significantly longer than the removal phase of the second male ($p<0.05$).

When data from the first and the second matings were combined, the average duration of the sperm removal phase was 2 min 10 s $\pm$ 1 min 15 s (range = 6 s – 4 min 46 s, n=36), which was equal to 23% of total copulation time ($\pm$ 8%, range = 2 – 40%, n=36).

3.1.4.2 Spermatophore transfer

To transfer spermatophores, the male swiftly moved his left fourth arm backward to a position just behind the funnel. At the same moment, a bundle of spermatopores was jetted out through the funnel's opening. When it protruded about three-quarter of the way out, the male quickly grabbed the bundle and trusted it forward into the female's buccal area. Each act of transfer took about 1-2 seconds. The grabbing action was accomplished by wrapping the hectocotylized portion (reduced suckers) of the left fourth arm around the spermatophores, forming a tight coil around them (Fig. 3.12). In 306 out of 307 acts of transfer observed, the males never failed to grab the spermatophores as they were protruding from the funnel. Only a single case of failure was observed, in which a male ejected a bundle of spermatophores prematurely, and could not grab it in time before it fell.

The internal process that initially propelled the spermatophores out through the funnel could not be observed directly, but presumably it may have involved the extension of the Needham's organ from inside the mantle cavity into the funnel.

During each transfer, an average of 9 ± 2 (range 5-14, n=34) spermatophores were ejected and transferred to the female's buccal area. No significant difference was found between the first and the second mating. The body size of the male and the size of Needham's organ did not affect the number of spermatophores ejected during each transfer.

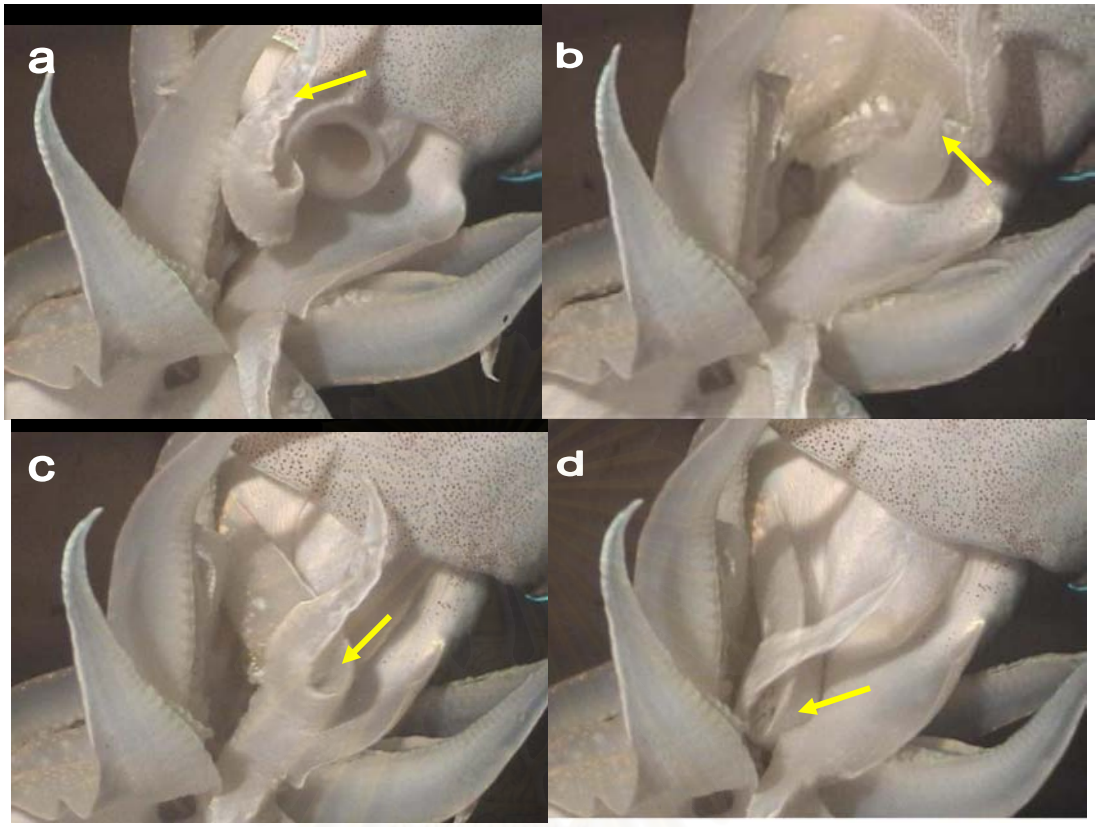


Fig. 3.12 a) to d) Chronological series of video captures showing the process of spermatophore transfer from a ventral view. a) Left fourth arm (arrow) is brought back toward the funnel. b) Several spermatophores are protruding from the funnel (arrow). c) The hectocotylized portion of the left fourth arm forms a loop (arrow) and grabs the spermatophores. d) The arm (arrow) trusts the spermatophores forward into the female's buccal area.

Spermatophore transfers occurred in sets. Each set always consisted of 2 consecutive transfers, one closely following the other. In a typical set of double transfers, the male first transferred a bundle of spermatophores, and then kept the distal portion of his left fourth arm in contact with the female's buccal membrane for about 9 seconds on average ($\pm$ 4 s, range 5-35 s, n=150), then swiftly retracted it to grab and transfer another bundle of spermatophores. This pattern ensued in all of the spermatophore transfers observed (n=307), except 2 incidents, in which the female broke off and jetted away before the male could transfer the second bundle of the set.

3.1.4.3 Spermatangia placement phase

After each set of spermatophore transfers, the male spent about 1 min 35 s on average in the phase called spermatangia placement (± 36 s, range 46 s – 4 min 32 s, n=113). During this phase, mostly the third and sometimes the fourth pair of arms of the male worked together in various combinations to press, squeeze, and grind the newly transferred spermatophores against the female's ventral buccal membrane, in order to achieve various tasks. The first task was to trigger the evagination of the spermatophores, which would then release the spermatangia that were inside. These spermatangia were then attached in place probably by the sticky substance that coated them, and with the help of manipulation by the male's arms. The suitable site for the deposition of spermatangia was typically on the inner side of the female's ventral buccal membrane (Fig. 3.13a). Although sometimes, the males accidentally attached some spermatangia to other places such as, on the other side or other regions of the female's buccal membrane (e.g. dorsal), on the female's arms (Fig. 3.13b), and on their own arms.

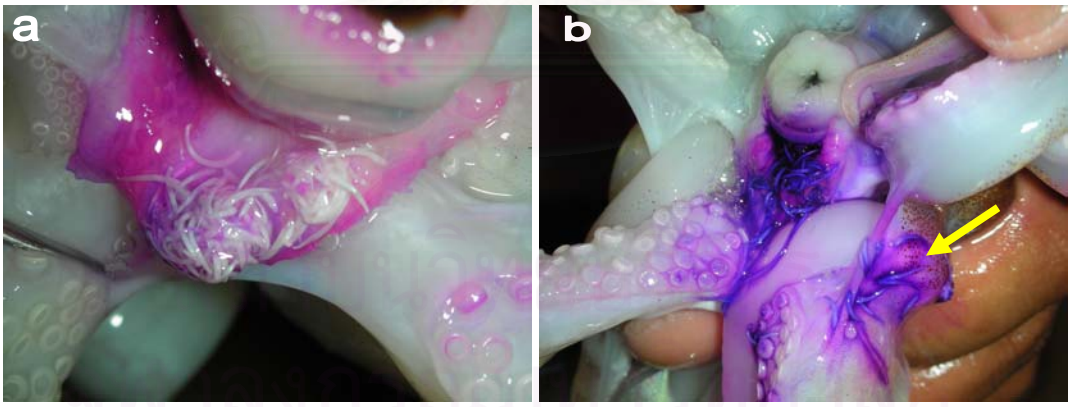


Fig. 3.13 a) Spermatangia placed on the inner surface of the ventral buccal membrane. b) Some spermatangia placed on the arms (arrow).

The average duration of this phase showed significant correlations to the overall duration of the copulation ($r = 0.85$, $p<0.05$, $n=34$, Fig.3.14a), and to the total number of

spermatophore transfers in the entire copulation ($r = 0.49$, $p<0.05$, $n=34$, Fig.3.14b). Hence, in longer copulations that involved more spermatophore transfers, the male also spent longer period of time in each placement phase. The average duration of this phase did not differ significantly between the first and the second mating. The size of the male and the size of its Needham's organ did not affect the duration of the phase.

After the spermatangia had been attached, the empty sheaths of evaginated spermatophore were blown away and discarded into the water column by jets of water pumped out through the male's funnel (Fig. 3.15a). The materials settled at the bottom of the tank after each mating trial consisted mostly of empty spermatophore sheaths (found in all 36 matings that were counted; mean $=55 \pm 29$, range 3-120, $n=18$, Fig. 3.15b). Of these, several sheaths often had spermatangia still attached to them, indicating that the male failed to attach them properly during the placement phase. In some cases (6 out of 36 matings counted), a small number of intact spermatophores were also found on the floor after mating.

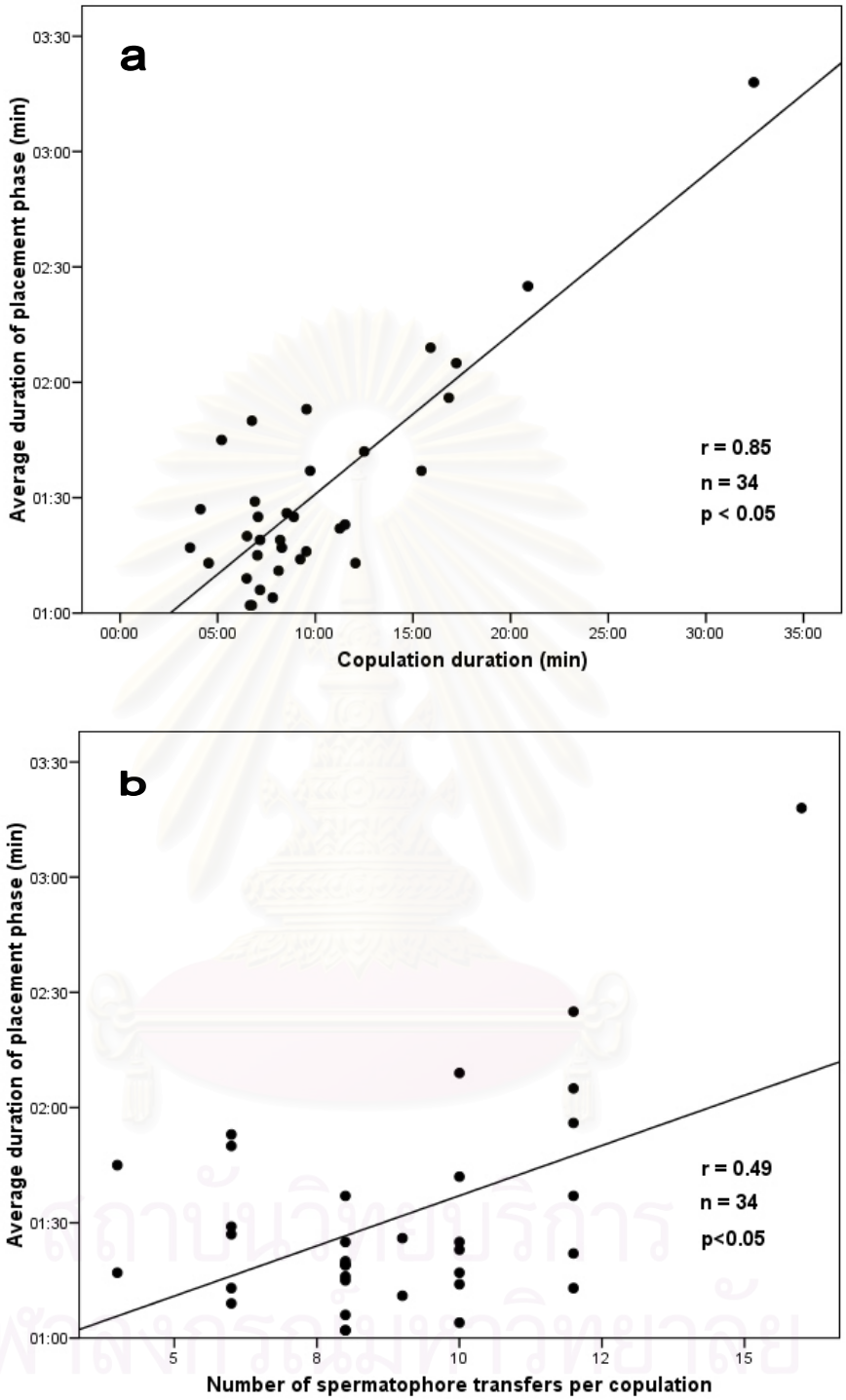


Fig. 3.14 Relationship between a) The average duration of the placement phase and the overall duration of the copulation. b) The average duration of placement phase and the number of spermatophore transfers during that copulation.

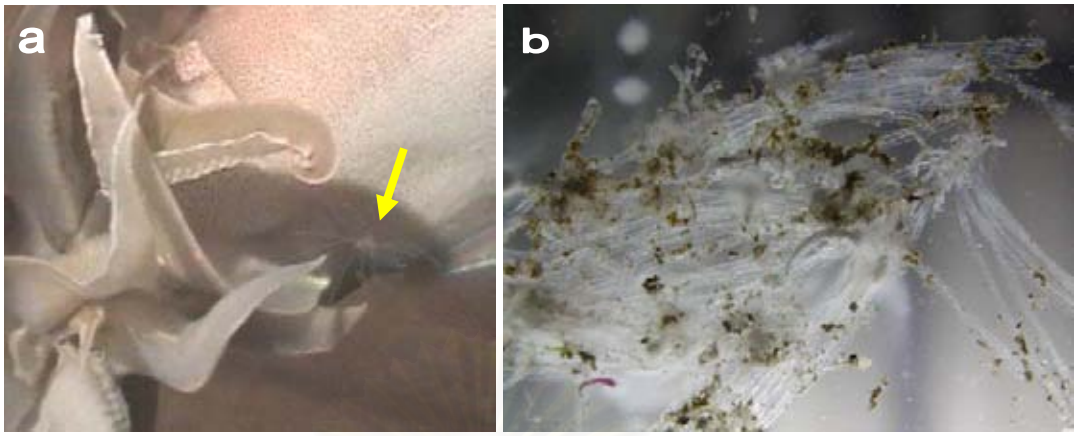


Fig. 3.15 a) Empty spermatophore sheaths (arrow) being blown out from the mouth area during mating. b) Spermatophore sheaths and other debris that have settled to the floor of the tank after mating.

3.1.4.4 Overall pattern of copulation sequence

During a typical copulation sequence, different phases were arranged in time as shown in Fig. 3.16. Copulation began with the removal phase, followed by multiple sets of double spermatophore transfers, each set separated by the spermatangia placement phase. In most cases, after the final placement phase, the male began to loosen its grasp gradually, and the copulating pair slowly became separated. However, in some cases, the copulation ended abruptly as the female jetted violently and broke away from the male. Immediately after the end of copulation, both individuals typically remained still at the bottom of the tank, while periodically jetting strong pulses of water through their funnels, rubbing their own arms against one another, and sometimes using some of the arms to reach into their own buccal area.

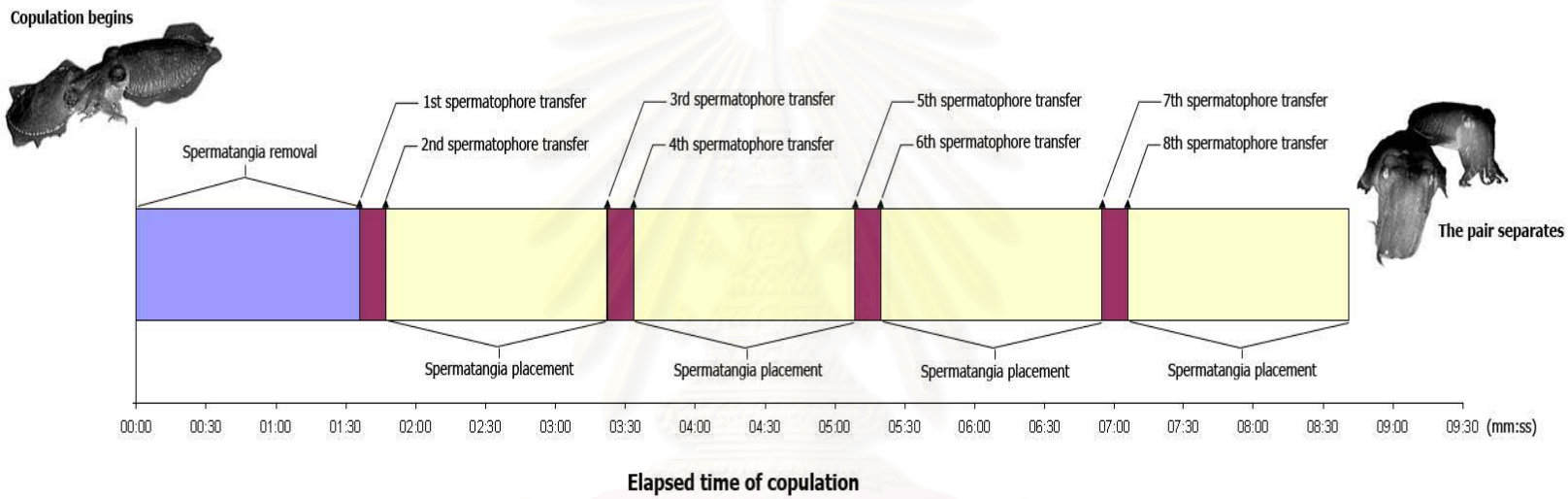


Fig. 3.16 A typical copulation sequence of *S. pharaonis*,

showing different phases, their average durations, and the order that they are arranged.

3.1.4.5 Number of spermatophore transfers per mating

During each copulation, the male transferred spermatophores as many as 8 times on average (± 3, range 3-16, n=36). When the first and the second matings were analyzed separately, the numbers of transfers per mating differed significantly ($p<0.05$). On average, the males that mated second (mean = 8 ± 2 transfers per mating, range 3-12, n=18) transferred 2 times less than the males that mated first (mean = 10 ± 3 transfers per mating, range 4-16, n=18). The number of transfers per mating was not affected by the sizes of the male, or the sizes of their Needham's organ.

3.1.4.6 Number of spermatophores transferred per mating

On average, a total of 77 spermatophores (± 28, range 16-165, n=34) were transferred by the male during a single copulation, without any significant difference between the first and the second mating. The number of spermatophores transferred per copulation correlated strongly with the number of transfers that took place during that copulation ($r = 0.69$, $p<0.0001$, n=34, Fig. 3.17), but it was not affected by the body sizes of the male or the size of their Needham's organ.

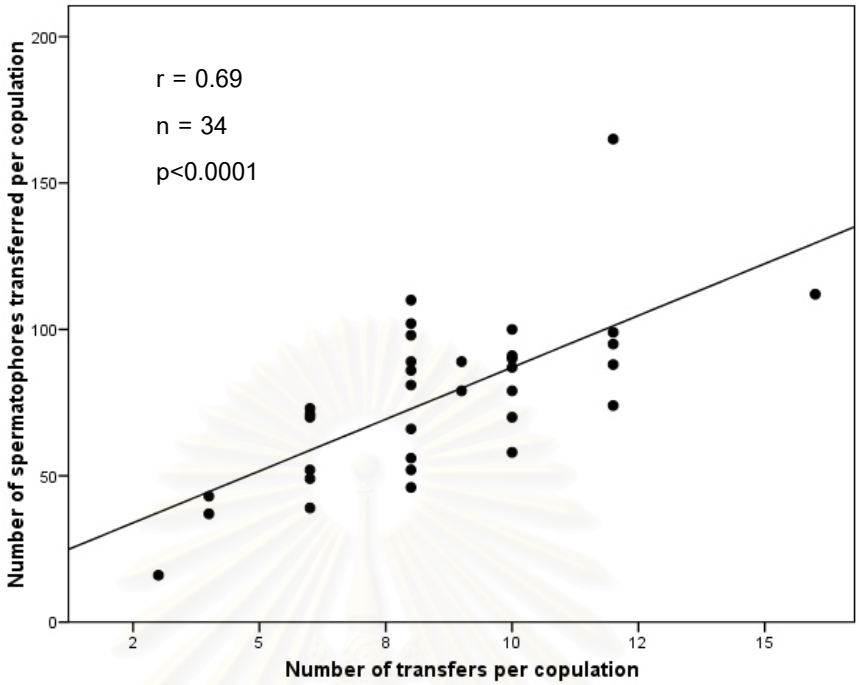


Fig. 3.17 Relationship between the number of transfers and the number of spermatophores transferred per copulation.

3.1.4.7 Copulation duration

The average duration of all copulations observed was 9 min 39 s (± 5 min 40 s, range 3 min 35 s – 32 min 27 s, n=36). When analyzed separately, the first mating lasted 11 min 52 s (± 6 min 24 s, range 3 min 35 s – 32 min 27 s, n=18), and the second mating lasted 7 min 26 s on average (± 3 min 49 s, range 3 min 40 s – 20 min 53 s, n=18). The average duration of the first mating was significantly longer than that of the second mating by 4 min 27 s ($p<0.05$). Overall, the duration of copulation was not affected by the body sizes of the males or their Needham's organ's sizes. It did, however, increased in relation to the number of transfers ($r=0.84$, $p<0.0001$, n=36, Fig. 3.18a) and the total number of spermatophores transferred ($r=0.36$, $p<0.05$ n=34, Fig. 3.18b) during the copulation.

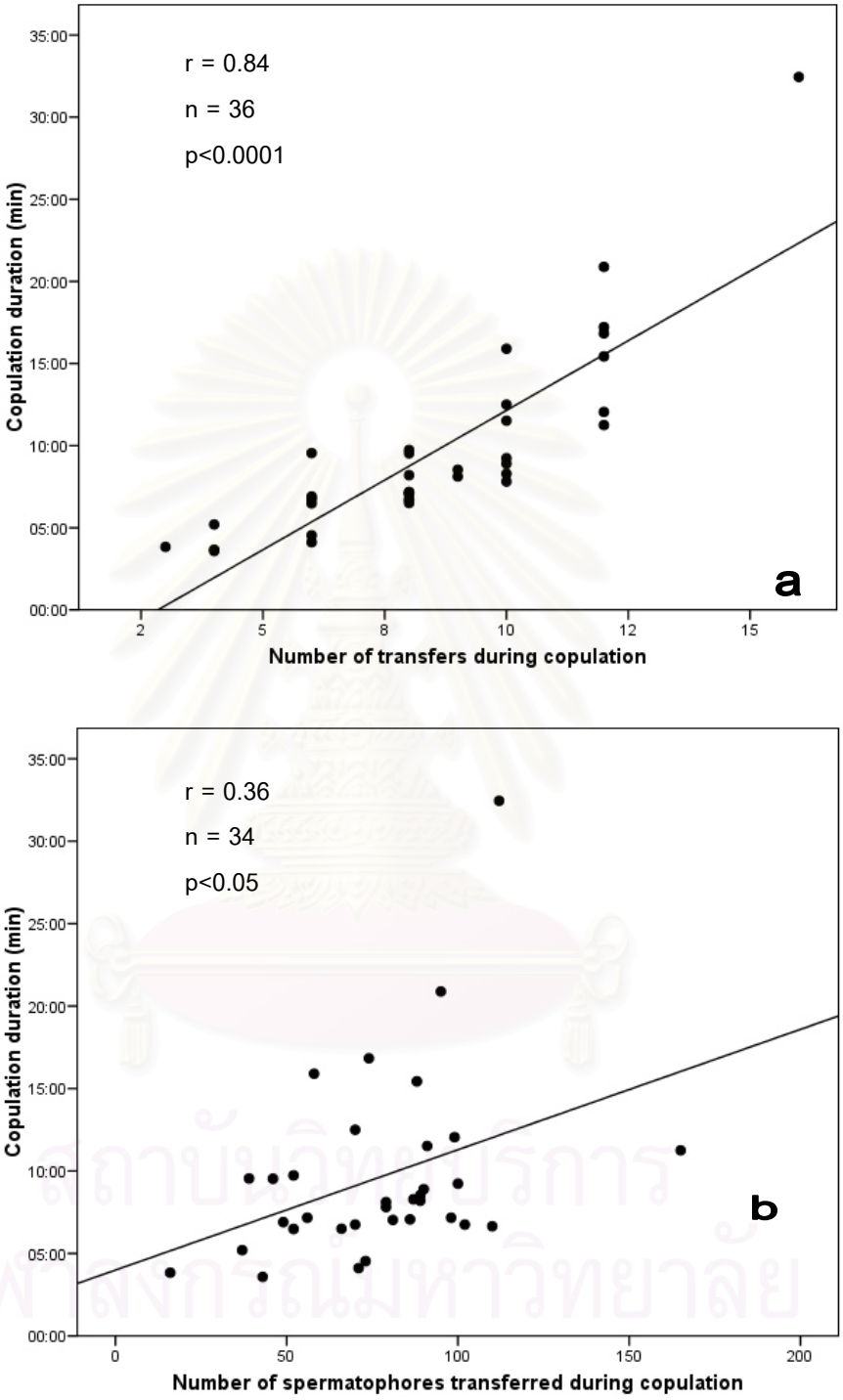


Fig. 3.18 Relationship between a) copulation duration and the number of transfers. b) copulation duration and the number of spermatophores transferred.

3.1.5 Factors affecting sperm removal

During the removal phase, the male removed on average 22 spermatangia, which was approximately 34% of all spermatangia that had been attached successfully by the previous male. The total number of spermatangia removed depended on the number of spermatangia that were present. There was a positive significant correlation between the number of spermatangia removed, and the number of spermatangia that had been previously attached by the first male ($r = 0.57$, $p<0.05$, $n=18$, Fig. 3.19a). However, the percentage of the spermatangia removed, remained roughly the same as the number of previously attached spermatangia increased ($r = -0.07$, $p>0.05$, $n=18$, Fig. 3.19b).

The number of spermatangia removed also increased with the amount of time that the male spent in the removal phase ($r = 0.50$, $p<0.05$, $n=18$, Fig. 3.20), although the percent removal did not show significant correlation. The duration of the removal phase itself was, in turn, significantly correlated to the overall duration of the copulation, and the duration of each placement phase (for the first mating $r = 0.79$, $p<0.01$, $n=18$, and $r = 0.68$, $p<0.01$, $n=18$ respectively, Fig. 3.21a-b; for the second mating $r = 0.82$, $p<0.01$, $n=18$, and $r = 0.56$, $p<0.05$, $n=18$ respectively, Fig. 3.22a-b). However, there was no significant correlation between the amount of time spent in the removal phase by the second male, and the number of spermatangia previously attached by the first male. The variation in the duration of the interval between the first and the second mating did not have significant effects on the number of spermatangia removed, the percent removal, and the duration of the removal phase. Analysis of other factors (see section 2.1.3 in Chapter II) that may have been related to the variability in the amount of sperm removed showed no significant correlation, both to the absolute number, and the percentage of spermatangia removed.

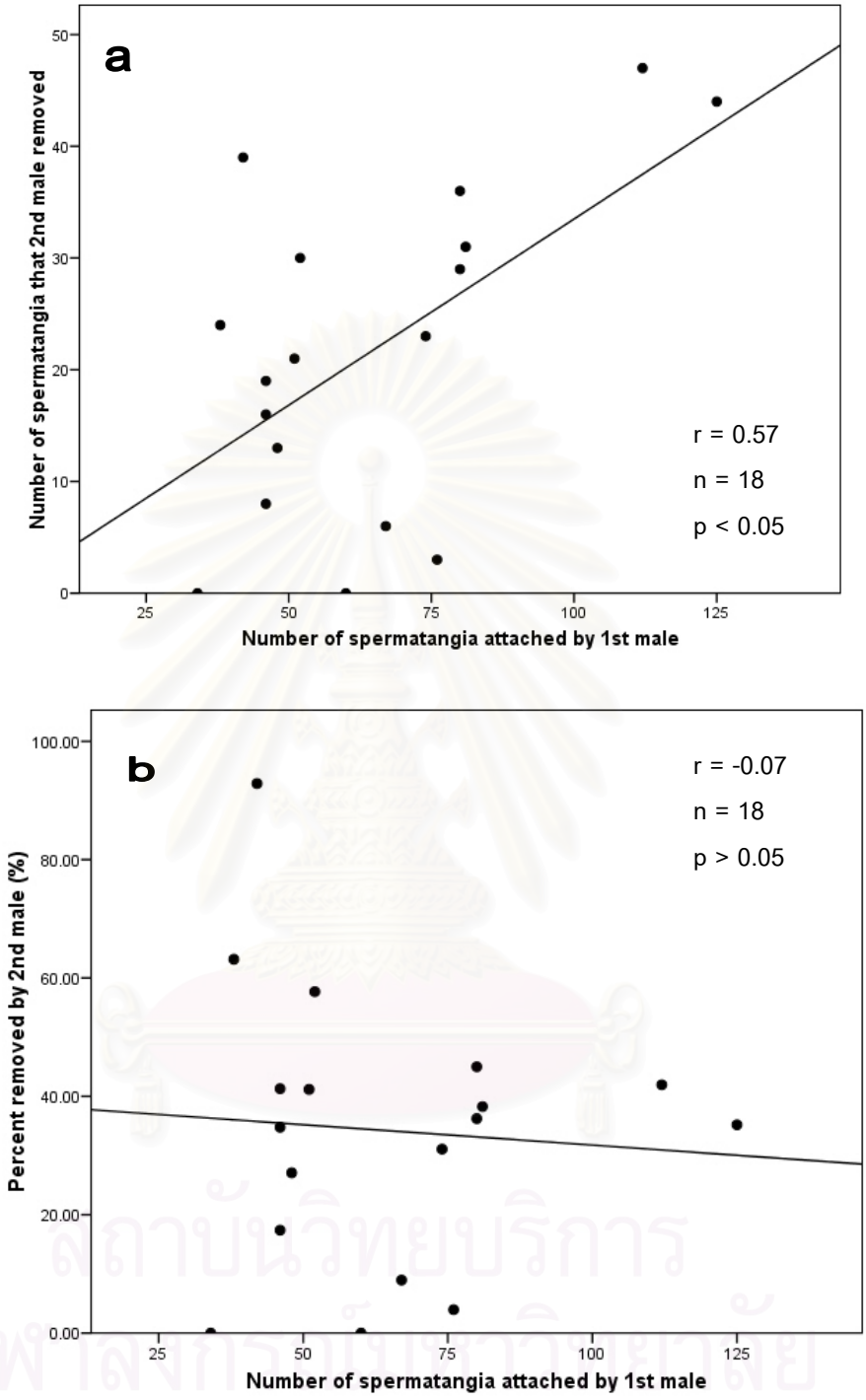


Fig. 3.19 Relationship between the number of spermatangia attached by the first male and a) the number of spermatangia removed by the second male, and b) the percentage of attached spermatangia that are removed.

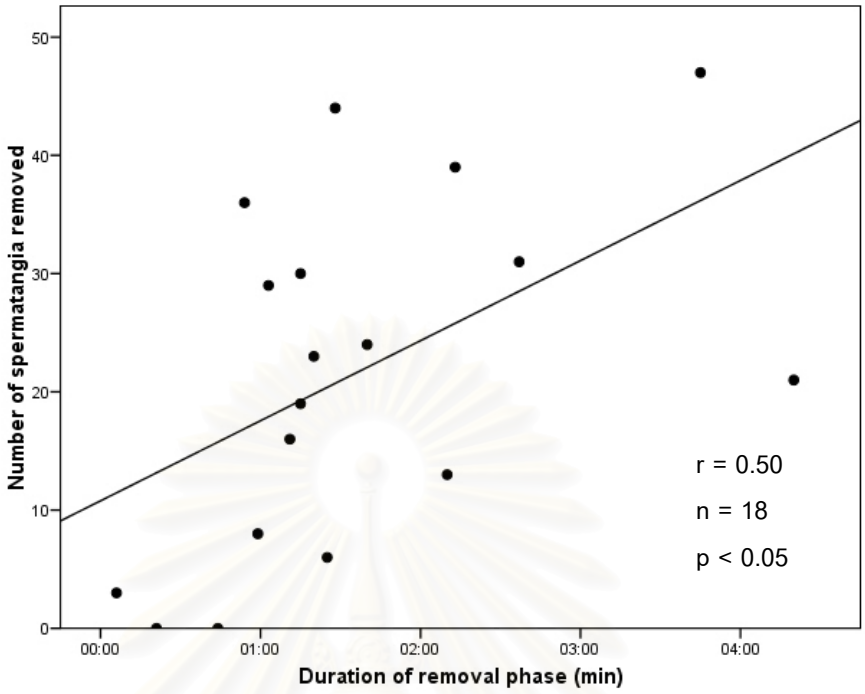


Fig. 3.20 Relationship between duration of the removal phase and the number of spermatangia removed.

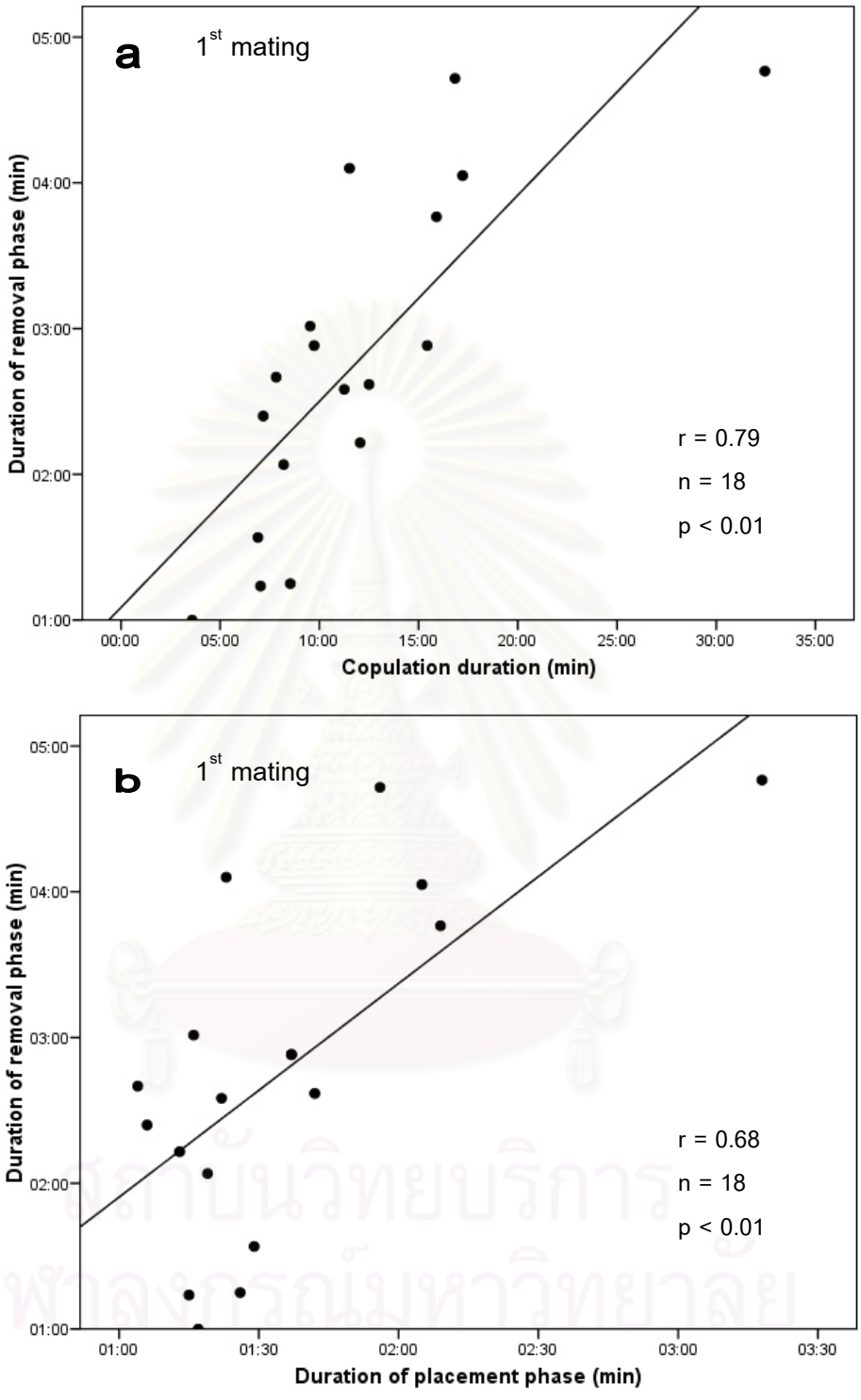


Fig. 3.21 Relationship between: a) Duration of the removal phase and the copulation duration (1st mating). b) Duration of the removal phase and the average duration of placement phase during the same copulation (1st mating).

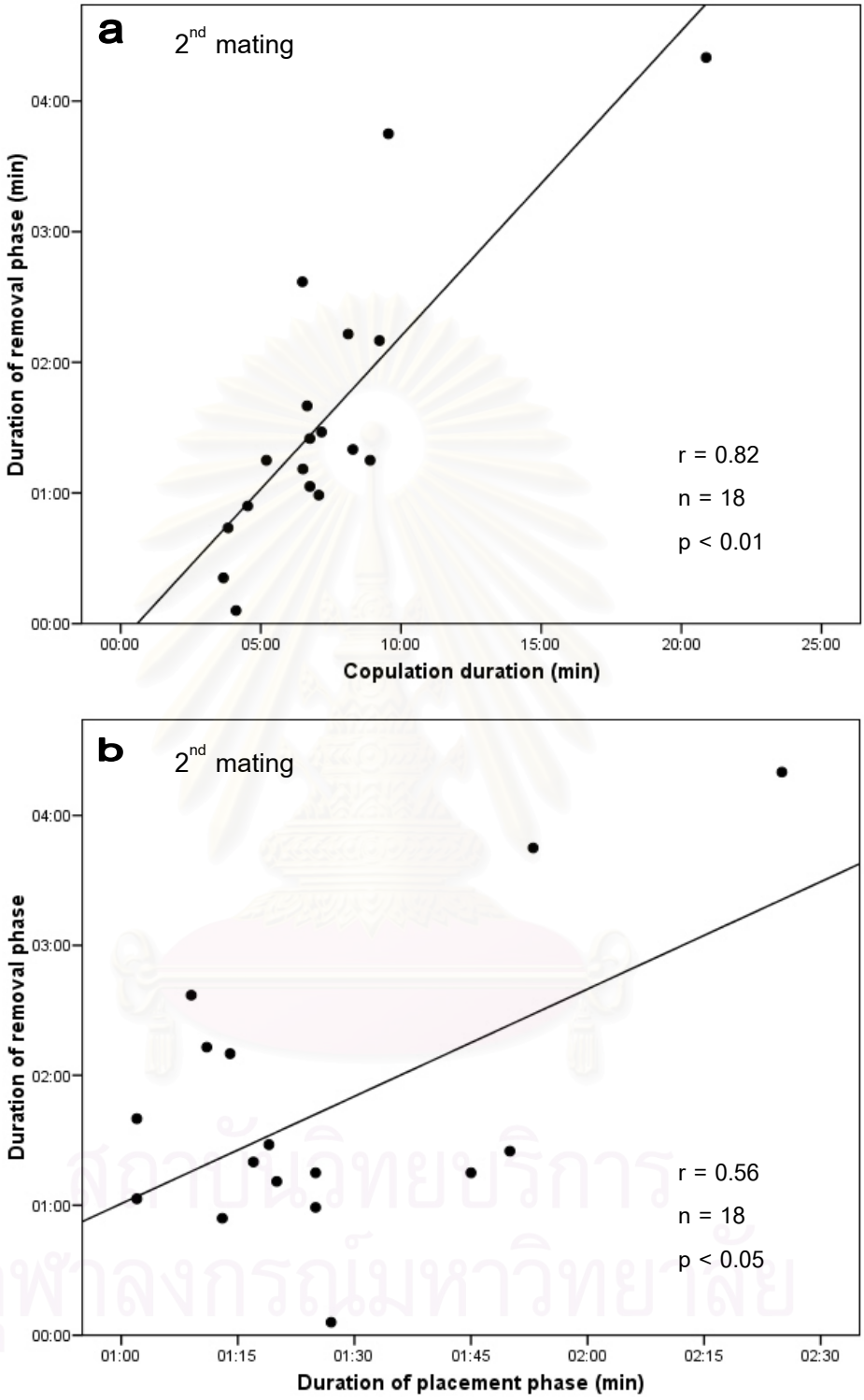


Fig. 3.22 Relationship between: a) Duration of the removal phase and the copulation duration (2nd mating). b) Duration of the removal phase and the average duration of placement phase during the same copulation (2nd mating).

3.1.6 Sperm removal by the female

Of all the 18 mating trials conducted during this experiment, one occurrence of sperm removal by the female was observed. After the attached spermatangia from the first mating were painted, and the particular female was released back into the observation tank, she began pulling some of these spermatangia out using the tips of her arms. Counts of colored spermatangia that fell to the floor before the second mating took place revealed a total of 8 spermatangia removed by the female. After this point, the second male successfully mounted and initiated copulation, and any further removal of spermatangia was done exclusively by him. The number of spermatangia removed by the female was taken into account while calculating the total number of spermatangia put out by the first male, but was not included while counting the number of spermatangia removed by the second male. In other trials, this behavior was not observed. However, the possibility that it was overlooked by the experimenter could not be ruled out completely.

3.1.7 Analysis of spermatangia attached on the female's buccal membrane

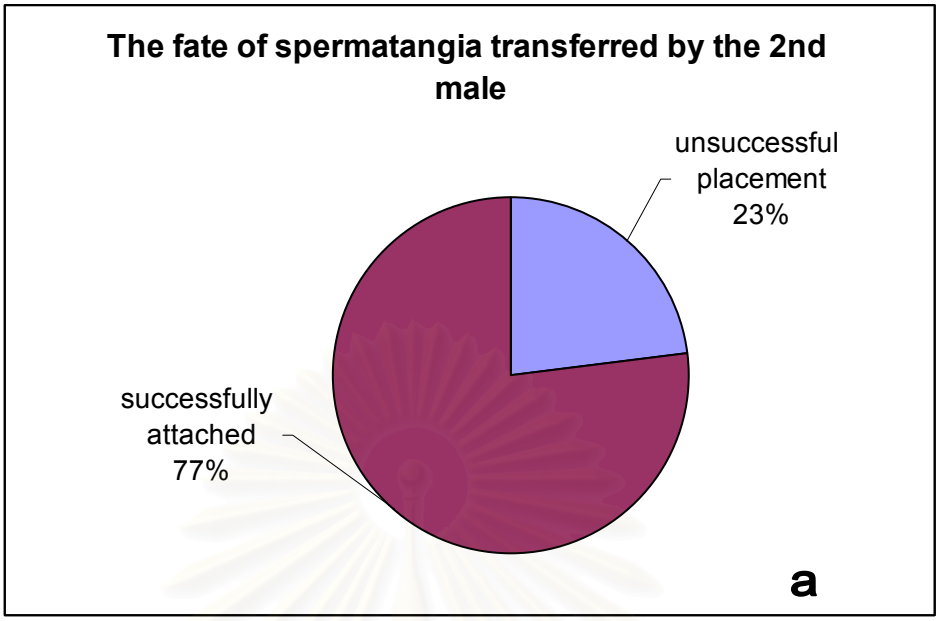
When there was no removal by the next male (second matings), an average of 23% of total spermatangia transferred were lost due to unsuccessful placement (most of these fell to the floor, and some were attached to the other place e.g. arms), and the remaining 77% were successfully attached on the female's ventral buccal membrane (Fig. 3.23a). When there was removal by the second male (first matings), an average of 29% of total spermatangia transferred were lost due to removal, 17% due to unsuccessful placement, and only 54 % were successfully attached on the female's ventral buccal membrane (Fig. 3.23b).

An average of 100 spermatangia ($\pm$ 32, range 38-174, n=18) were attached to the female's buccal membrane after the two copulations. Of these, an average of 43

were deposited by the first male, and 57 by the second male. The mean ratio between the two was 1 : 1.3.

In 11 out of 18 samples counted, the number of spermatangia from the second male exceeded the number of those from the first male. Of these, there were 4 cases in which, if there had not been any removal by the second male, the number of spermatangia attached by the first male would have actually exceeded that of the second. When such scenario (no removal) was applied to all cases, the ratio between the number of the first male's spermatangia and the number of the second male's spermatangia was shifted to 1.1 : 1, or 53% to 47%. Therefore, in terms of number of spermatangia attached to the female's buccal membrane, the second male had a slight advantage over the first male because of the removal behavior. Meanwhile, if there were no removal, the first male would be the one who had a slight advantage over the second male.

In 10 trials done in the later half of the experiment, the female's buccal membranes were examined more carefully, and in 7 of these trials, there were several (2-10) spermatangia found hidden inside the cavity of the seminal receptacles. These spermatangia were not visible at first, but when pressure was applied on the membrane where the openings of the receptacles lied, their tips began to emerge through the pores, and eventually the whole spermatangia could be pulled or squeezed out (Fig. 3.24). The origin of these spermatangia, which appeared white, could not really be assigned to either the first or the second male. They may as well had come from other previous matings that the female had had in the wild before it was captured. Due to the uncertainty, their numbers were, therefore, not included in any of the calculations.



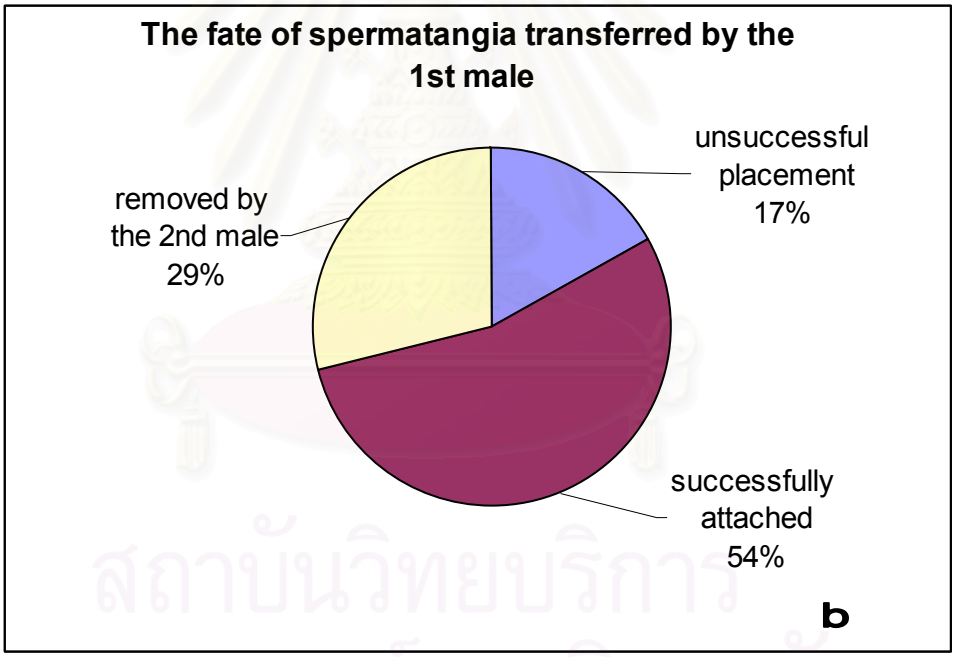


Fig. 3.23 Percentage of spermatangia that resulted in different fates. a) The 2nd male's spermatangia. b) The 1st male's spermatangia.

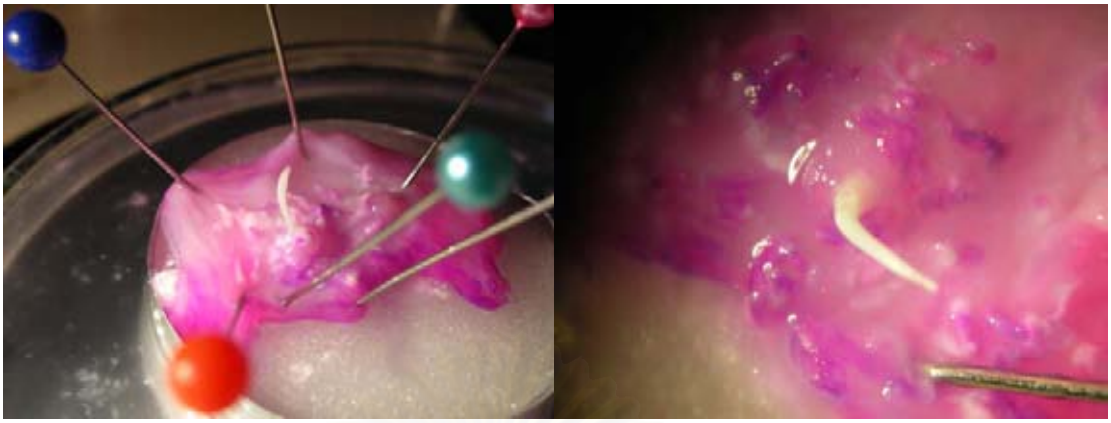


Fig. 3.24 Whole spermatangia were sometimes found hidden inside the cavity of seminal receptacle.

3.2 Experiment II

3.2.1 Mating duration

The average duration was 12 min 17 s (± 3 min 24 s, n = 14) for the first mating, and 10 min 24 s (± 5 min 4 s, n = 14) for the second mating, without a significant difference between the two averages. Among the 14 trials, the interval between two matings ranged from 4 minutes to 5 days (about 5-10 min in 6 trials, 1-4 h in 3 trials, and 1-5 days in 5 trials, Table 3.1). The wide range in time interval was due to the unpredictable nature of the males. The second male was always released to the female right after the end of the first mating. Sometimes they mated almost immediately, hence the trials with shorter intervals between the two matings. But sometimes, the male did not show an interest to mate, and after several hours, he had to be taken back to his tank, and the mating had to be postponed to the next day, hence the trials with longer intervals. The variation in the length of interval did not show a significant correlation with the duration of the second mating that followed.

Table 3.1 Duration of 1st and 2nd mating, and the duration of interval that elapsed between them.

Trial#	Male ID, first/second	Female ID	1st mating duration	Interval	2nd mating duration
1	MA / MD	Fe5	13 m	2 h 35 m	6 m
2	MD / MA	Fe4	11 m	4 h 14 m	10.5 m
3	MB / MC	Fe3	14 m	5 m	9 m
4	MC / MB	Fe6	8 m	4 m	9 m
5	M1 / M4	F1	16 m	1 h 7 m	7 m
6	Br / M3	F7	6 m	7 m	6 m
7	Br / M1	F3	17 m	5 d	8 m
8	M7 / M14	F13	10 m	5 m	26 m
9	M5 / M7	F8	15 m	5 d	11 m
10	M13 / M11	F11	17 m	5 m	11 m
11	M6 / M9	F18	11 m	10 m	11 m
12	M14 / M7	F15	14 m	26 h	13 m
13	M9 / M6	F17	11 m	25 h	6 m
14	M11 / M9	F16	9 m	28 h 20 m	12 m

Abbreviations: (d) = days, (h) = hours, (m) = minutes, and (s) = seconds.

3.2.2 Fecundity and hatching rate

Of all 14 females used in the experiment, 12 subsequently laid eggs (2 died before they spawned). The female attached the eggs one at a time to a substrate, which could be either the wall of the tank, the drain pipe, the bottom of the floating basket, or the provided netting and mesh. The deposited eggs always formed a single cluster, except in one case, in which the female was blind (due to eye infection) and attached the eggs all over the place around the tank. Of the 12 females that laid at least one

clutch of eggs, data were successfully collected from 11 of them. Of these 11 females, 10 subsequently laid a second clutch of eggs, and of these, 5 still went on to lay more clutches before they died (maximum = 4 clutches). After the second clutch, data were collected only from the third clutch, and only from 3 individual females.

The first clutch of eggs was laid ranging from on the same day as the second mating to 12 days after the second mating (mean=3.6 ± 3.6 d, n=11). The first clutch contained 329 eggs (± 136, range 30-490, n=11), which took 15 days to incubate (± 1.4 d, range 13-17 d, n=10). The hatching rate was 0 % in one case, and the rest ranged from 3% to 88%, with an average of 47% (± 31%, n=10). The eggs that did not reach the hatching stage, in some cases, did not develop from the beginning because they might have not been fertilized. In other cases, the eggs did develop to a certain stage before their development became arrested. The abnormal development and the low hatching rate may have been caused by fungal or algal infection of the eggs, or brief changes in water salinity and temperature, as suggested by Nabhitabhata and Nilaphat (1999).

The second clutch of eggs was spawned 5.4 days after the first clutch was spawned (+ 1.6 d, range 3-8 d, n=10). It contained 338 eggs (± 203, range 81-649, n=10), and took 14 days to hatch (± 1.9 d, range 12-17 d, n=9). The hatching rate was 0% in one case (laid by the same female whose first clutch also had 0% hatching rate), but for the rest, it ranged from 10% to 89%, with an average of 48% (± 31%, n=9).

The third clutch of eggs was spawned 5.4 days after the second clutch (+ 1.6 d, range 4-8 d, n=5). It contained on average 173 eggs (± 91, range 70-243, n=3), and took 15.5 days to incubate (n=2). The hatching rate was 0% in one case, which again belonged to the female whose first and second clutch also did not hatch. For the remaining 2 cases, the hatching rates were 27% and 35% (mean = 36%).

Table 3.2 The number of eggs contained in each clutch, the number of eggs hatched, the incubation period, hatching rate (%), and the number of days between spawnings.

Trial#	No. of clutches laid after mating	1st clutch					2nd clutch					3rd clutch				
		Days after last mating	Incubation period (days)	Numbers of eggs laid	Number of eggs hatched	Hatching rate (%)	Days since last clutch	Incubation period (days)	Numbers of eggs laid	Number of eggs hatched	Hatching rate (%)	Days since last clutch	Incubation period (days)	Numbers of eggs laid	Number of eggs hatched	Hatching rate (%)
1	2	1	13	378	191	51	5	12	290	258	89	-	-	-	-	-
2	2	6	15	490	432	88	6	14	649	482	74	-	-	-	-	-
3	2	1	15	425	255	60	4	16	161	90	56	-	-	-	-	-
4	2	3	17	292	9	3	4	17	600	77	13	-	-	-	-	-
5	4	Data not collected														
6	0	Female died before laying eggs														
7	2	5	15	346	76	22	6	16	81	15	19	-	-	-	-	-
8	4	2	14	293	76	26	8	13	483	255	53	5	15	70	31	44
9	0	Female died before laying eggs														
10	1	6	14	30	21	70	-	-	-	-	-	-	-	-	-	-
11	3	12	17	142	10	7	3	15	117	12	10	-	-	-	-	-
12	4	0	15	423	273	65	5	12	199	168	84	4	16	205	55	27
13	3	0	-	381	0	0	8	-	464	0	0	8	-	243	0	0
14	3	4	13	421	351	83	5	12	339	122	36	-	-	-	-	-
average		3.6	15	329		47	5.4	14	338		48	5.4	15.5	173		36

There was no significant difference between numbers of eggs the same female laid during the first and the second spawning. The hatching rate also did not differ significantly across the first and the second clutch laid by the same female. Across individuals, there was no significant correlation between the number of eggs laid in the first clutch, and the number of eggs laid in the second clutch. There was however, a significant correlation between the hatching rate of the first and the second clutch

(r=0.674, p=0.047, n=9). Comparison with the third clutch was not analyzed statistically because the sampling size was not great enough.

3.2.3 ISSR-PCR primer screening

Forty eight ISSR primers (Appendix A) were tested using DNA samples from 16 males and 18 females. Fifteen primers produced clear banding patterns (17899A, HB12, HB13, HB15, SAS1, SAS3, T8707, UBC813, UBC824, UBC826, UBC845, UBC868, 814, 844A, 844B). However, only 6 of these revealed polymorphisms that were potentially usable for the analysis of paternities in this study (SAS1, UBC813, UBC824, UBC868, 814, HB13). Furthermore, the parents of each family (the female and the 2 potential fathers) differed only with respect to 2-4 of the primers, and not all 6 of them (Table 3.3). Three families (Br-M1-F3, M13-M11-F11, and M11-M9-F16), each consisted of the parents along with 10-16 offspring sampled from the same clutch have been tested for DNA fingerprints using the selected potential primers. For each family, only 1-2 primers actually yielded scorable and reproducible banding patterns (Appendix B). Some of the primers that used to produce clear bands during previous testings failed to generate the same clear patterns in these final tests. In conclusion, the number of useful and reliable primers found during this study was not great enough for carrying on further analysis, hence paternities of the hatchlings were not successfully assigned, and the sperm precedence pattern in *S.pharaonis* remained to be elucidated.

Table 3.3 ISSR primers selected for testing DNA fingerprints for each family.

Trial #	Parents (first male-second male-female)	Primers that revealed polymorphism among the parents
7	Br-M1-F3	814, SAS1
8	M7-M14-F13	SAS1, UBC813, UBC824
10	M13-M11-F11	SAS1, UBC813, HB13
11	M6-M9-F18	SAS1, UBC868, UBC824, hb13
12	M14-M7-F15	SAS1, UBC813, UBC824
13	M9-M6-F17	SAS1, UBC868, UBC824, hb13
14	M11-M9-F16	UBC813, UBC824, UBC868

3.3 Experiment III

3.3.1 Mating and re-copulation

Out of 25 trials conducted (Table 3.4), 18 resulted in at least one mating. Of these, there were 15 trials, in which the male mated the second time within the time limit of the observation. And out of these 15 trials, 14 trials showed the male copulated for the second time consecutively with the same female. Only 1 trial showed the male switching, and carried out his second copulation with the other female he had not mated. Within the 14 trials in which re-copulation occurred, the interval between the first and the second copulation varied from 1 min to 2 h 30 min (mean = 47 ± 47 min, n=13). The first copulation took 5 min 38 s (n=13), while the second copulation took 2 min 5 s (n=13) on average. The first copulation's duration was significantly longer than the second one's ($p<0.05$, n=13). Positive significant correlations were also found between each pair of the following factors; the duration of the first copulation, the interval, and the duration of the second copulation (Fig. 3.24 – 3.25).

Table 3.4 Mating results of trials in experiment III. (cop) = copulation.

Trial #	Male ID	Female ID	1st cop with	2nd cop with	Re-copulation
1	mA	fA, fB	fB	fA	No
2	mA	fA, fB	fB	fB	Yes
3	mB	fA, fB	fA	fA	Yes
4	mC	fB, fC	fC	fC	Yes
5	mD	fB, fC	-	-	-
6	mD	fB, fE	fE	fE	Yes
7	mD	fB, fE	fB	-	-
8	mD	fB, fE	fB	-	
9	mE	fD, fE	fE	fE	Yes
10	mF	fB, fE	-	-	-
11	mF	fB, fE	-	-	-
12	mF	fB, fE	-	-	-
13	mG	fB, fC	-	-	-
14	mG	fB, fE	fE	fE	Yes
15	mG	fB, fE	fB	-	-
16	mG	fB, fE	fB	fB	Yes
17	mH	fB, fE	fE	fE	Yes
18	mH	fB, fE	fB	fB	Yes
19	mH	fB, fE	fB	fB	Yes
20	mI	fB, fC	fC	fC	Yes
21	mI	fB, fC	fC	fC	Yes
22	mJ	fB, fC	-	-	-
23	mJ	fB, fE	fE	fE	Yes
24	mJ	fB, fE	fE	fE	Yes
25	mJ	fB, fE	-	-	-

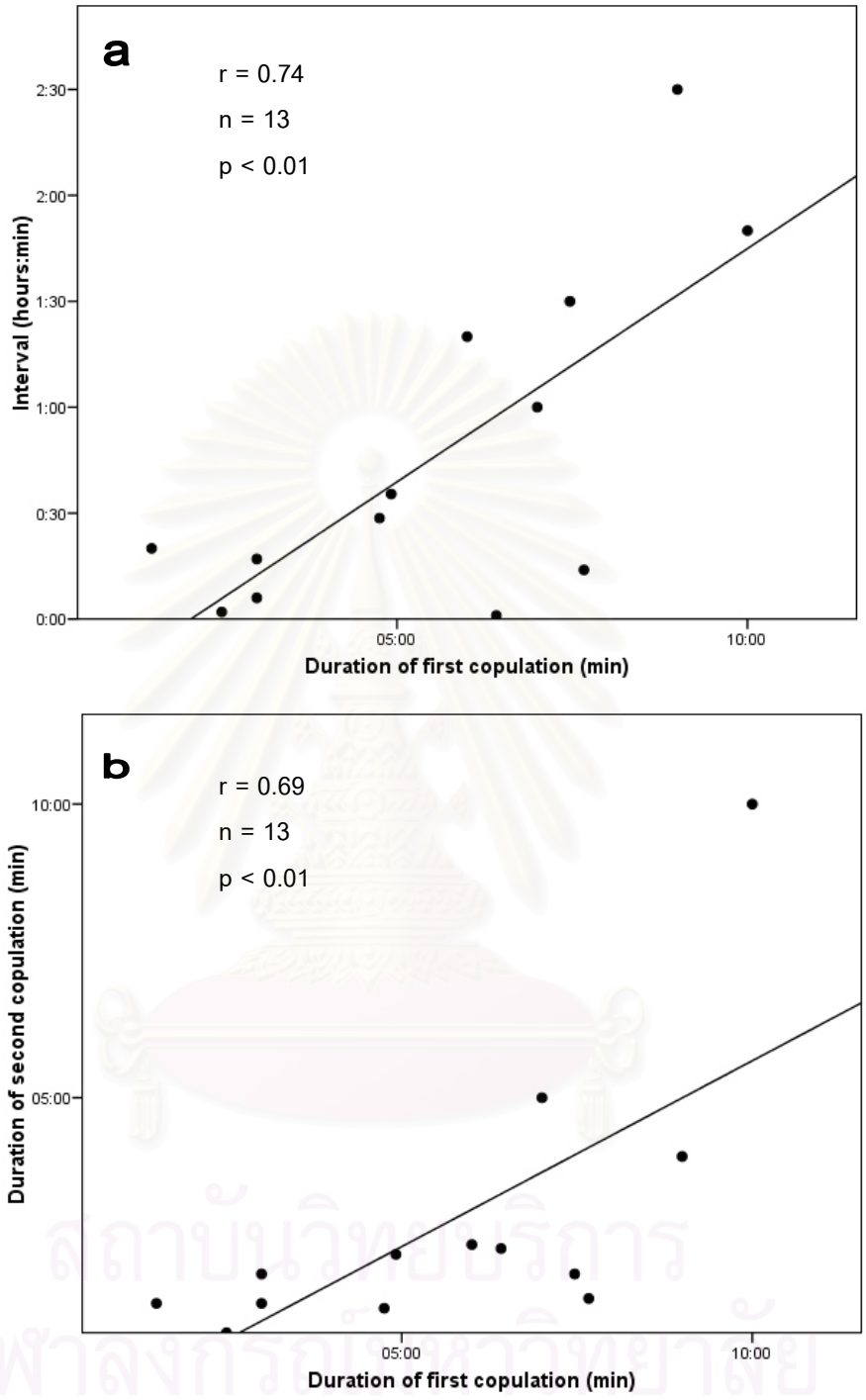


Fig. 3.24 Relationship between duration of the first copulation and a) the time elapsed until the second copulation (interval), and b) duration of second copulation.

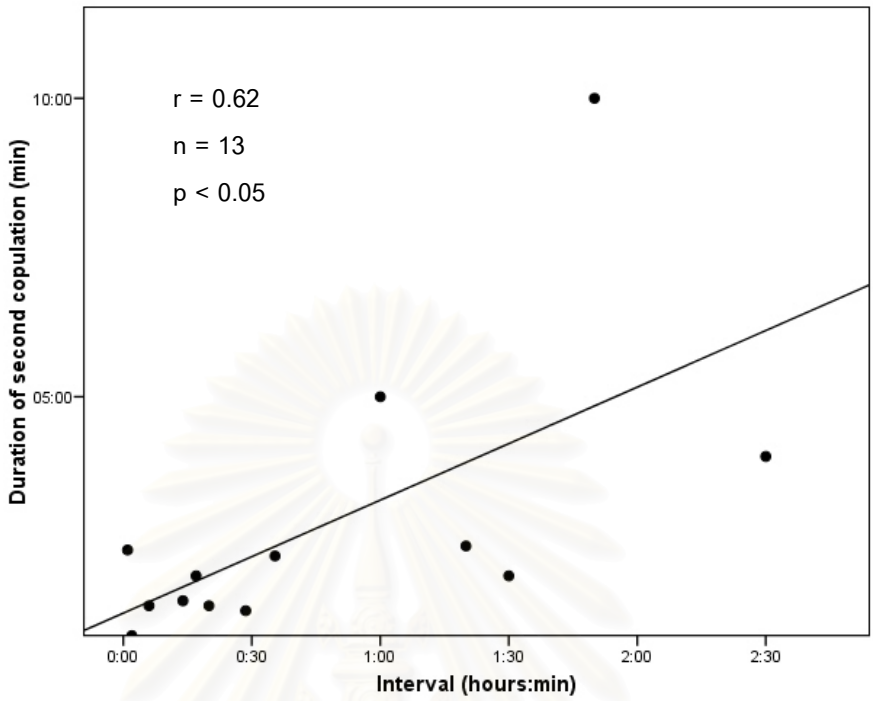


Fig. 3.25 Relationship between the interval and second mating's duration.

In some of the trials, the males were tested repeatedly on three different dates, using the same pair of females (e.g. fB and fE). Each male's choices of mate were not necessarily consistent across the dates (Table 3.5). This suggests that the male may choose the female either randomly, or based on certain qualities that are dynamic, such as her behavioral receptivity at that particular point in time, rather than the qualities that are permanent, such as her body size. Moreover, the males' choices of mate in trials conducted on the same day were not necessarily consistent across individuals. This, again, suggests that the male's choice of mate could be random, or could be based on certain qualities of the female that fluctuate quite often, even within the same day.

Table 3.5 Results of trials that used the same male, paired with the same 2 females (fB and fE), but conducted on different dates. (nm) = no mating.

Date / Male ID	1st copulation with / 2nd copulation with		
	13/09/05	14/09/05	18/09/05
mD	fE/fE	fB/nm	nm
mG	fE/fE	fE/fE	fB/fB
mH	fE/fE	fB/fB	fB/fB
mJ	fE/fE	fE/fE	fE/fE

3.3.2 Pre-copulatory behaviors

3.3.2.1 Female rejection

A female was considered rejecting a male when she tried to flee from his approach. More subtly, she may have allowed the male to get near, but put up a resistance only when he tried to grasp or force her into the copulating position. The female's rejecting behavior, as observed in this study, was typically exhibited in association with one or more of the following components summarized in Table 3.6 (see also Fig. 3.26).

When the female did not exhibit rejection, she remained still and passively allowed the male to copulate (sometimes, the pair also engaged in parallel synchronized swimming, by positioning their bodies side by side and moving back and forth together). The color patterns and posture of the female during this period did not differ from those exhibited when she swam alone under a normal circumstance (cryptic pattern, arms stretched forwards).

Table 3.6 Signal components of female rejection in *S. pharaonis*

Type of signal components	Descriptions
Arm posture	1st arms raised straight up, bilateral. One of 4th arms point toward the male, unilateral.
Color pattern on the body	Often dark brown, but sometimes pale. Big white band across mid-body, unilateral.
Color pattern on the head	Dark eye rings.
Color pattern on the arms	4th arm white, unilateral.
Fins	Dark or clear.
Mantle edge	White, with or with out interspersed black stripes.
Orientation / movements and other behaviors	Jetting away from the male. Inking.
Skin texture	Smooth or rough.

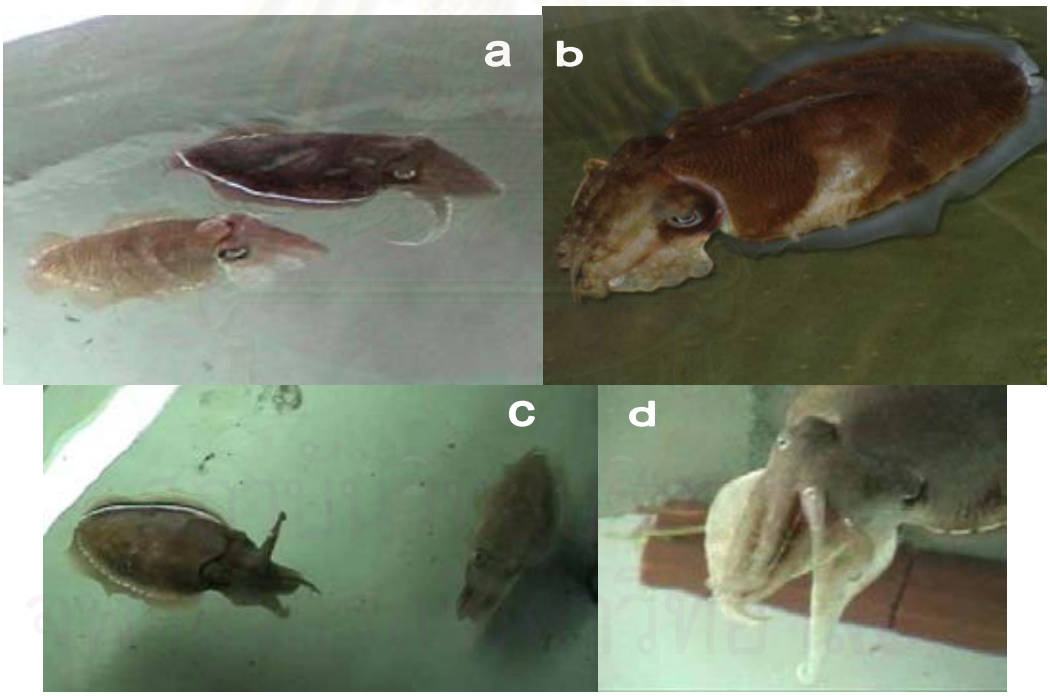


Fig. 3.26 Female rejection in *S. pharaonis*. a) Fourth arm is often pointed toward the male. b) Arms appear white, dark rings from around the eyes, a big white band appears unilaterally across the body. c) and d) When very agitated by the male (on the right in c), the female often raises the first pair of arms upward.

3.3.2.2 Male courtship and initiation of copulation

Upon detecting a female's presence, the male typically approached her slowly while putting on a distinct display. The color patterns on the body and the arms abruptly changed from pale or camouflage to orange background with dark tiger stripes on it. At the same time, dark rings formed around the eyes, and underneath that, the colors of the skin around the cheek area became much more greenishly iridescent than usual. All the arms were pointed downward or slightly forward, and they were all flattened and expanded (especially the 4th arms), giving the male the appearance of having a very big round face. Thus, this particular display was named the Round Face Tiger Display in this study (Table 3.7 and Fig. 3.27). The Round Face Tiger Display was, in many respects, similar to the agonistic Intense Zebra Display (Fig. 3.28) exhibited by the male of many cuttlefish species including *S. pharaonis*. However, it did differ distinctively in that the background color of the skin was orange instead of white, and that the contrast level between the stripes and the background was much lower, compared to that of the Intense Zebra Display.

Table 3.7 Signal components of The Round Face Tiger Display in *S. pharaonis*

Type of signal components	Descriptions
Arm posture	All point downward, no spread, each expanded, flattened, especially 4th arms.
Color pattern on the body	Tiger stripes on orange background.
Color pattern on the head	Forehead same as body, dark rings around the eyes, greenish iridescence on the cheeks.
Color pattern on the arms	Tiger stripes, high contrast.
Fins	Clear.
Mantle edge	White, interspersed with black stripes.
Orientation / Movements and other behaviors	Approach, face toward the side of female. Or face the same direction as female, body parallel, slightly behind.
Skin texture	Smooth.

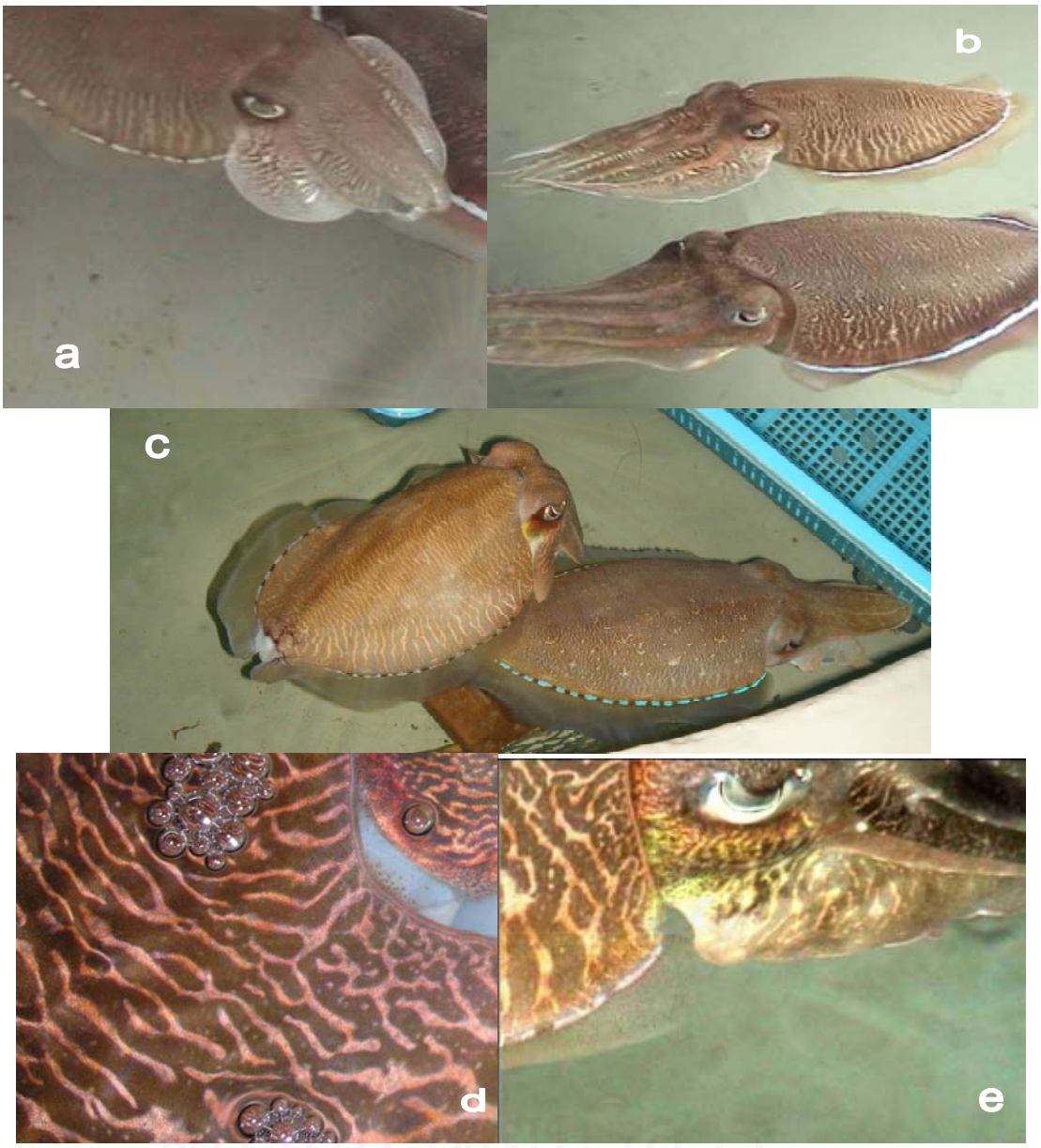


Fig. 3.27 The male's Round Face Tiger Display in *S. pharaonis*. a) to c) The male positions himself next to or above the female. Arms are striped, extended, and flattened, giving the appearance of having a big round face. d) Mantle shows tiger pattern with orange background. e) The cheek area becomes greenishly iridescent.

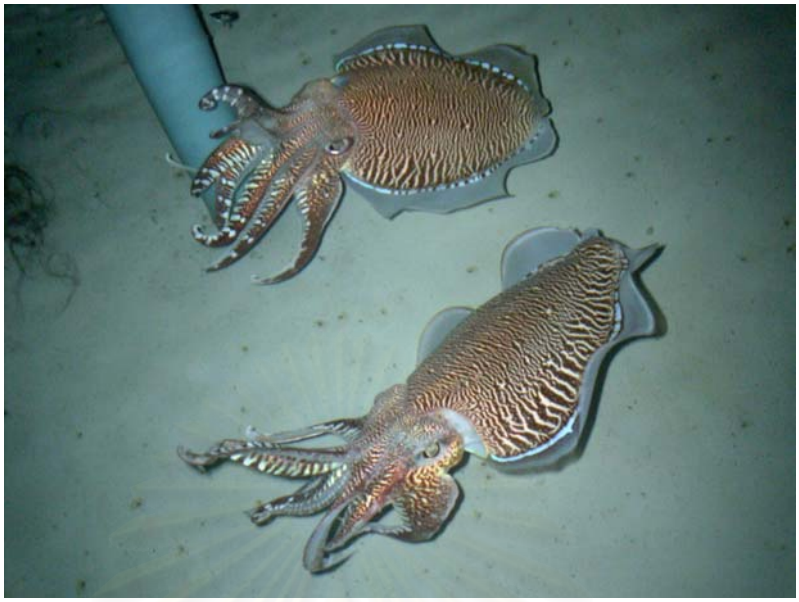


Fig. 3.28 Two *S. pharaonis* males engaging in agonistic interactions, showing off their Intense Zebra Display. Notice that the contrast level of the stripes is higher than in the Round Face Tiger Display, and the background appears whitish rather than orange.

Initially, while approaching the female, the male typically presented his face toward her. Later on, he rotated to align his body parallel to the female. At this point, if the female showed no sign of rejection, or only showed weak rejection, the male typically proceeded to position himself on top of her, and touched the female's dorsal with the underside of his arms. To initiate copulation, the male slid forward so that his arms were placed directly on top of the female's arms. At this point the male briefly exhibited another distinct display, called in this study, the Buffalo Display, in which he stretched out his fourth arms to its maximum length and spread them to the sides, creating a resemblance to the buffalo horns (Table 3.8 and Fig. 3.29). During the display, the male used the rest of the arms (the first, second, and third pairs) to forcefully grasp the female's arms from above, and then turned his body around into the head-to-head position, with all his arms intertwined with those of the female (Fig. 3.30). Copulation typically ensued from this point on.

Table 3.8 Signal components of the Buffalo Display in *S. pharaonis*

Type of signal components	Descriptions
Arm posture	1st, 2nd and 3rd arms point downward, 4th arms extend and spread to the sides.
Color pattern on the body	Tiger stripes on orange background.
Color pattern on the head	Forehead same as body, dark rings around the eyes, greenish iridescence on the cheeks.
Color pattern on the arms	Very dark tiger stripes, very high contrast.
Fins	Clear.
Mantle edge	White, interspersed with black stripes.
Orientation / Movements and other behaviors	On top of female, facing the same direction, all arms except 4th trying to grasp the female's arms.
Skin texture	Smooth.

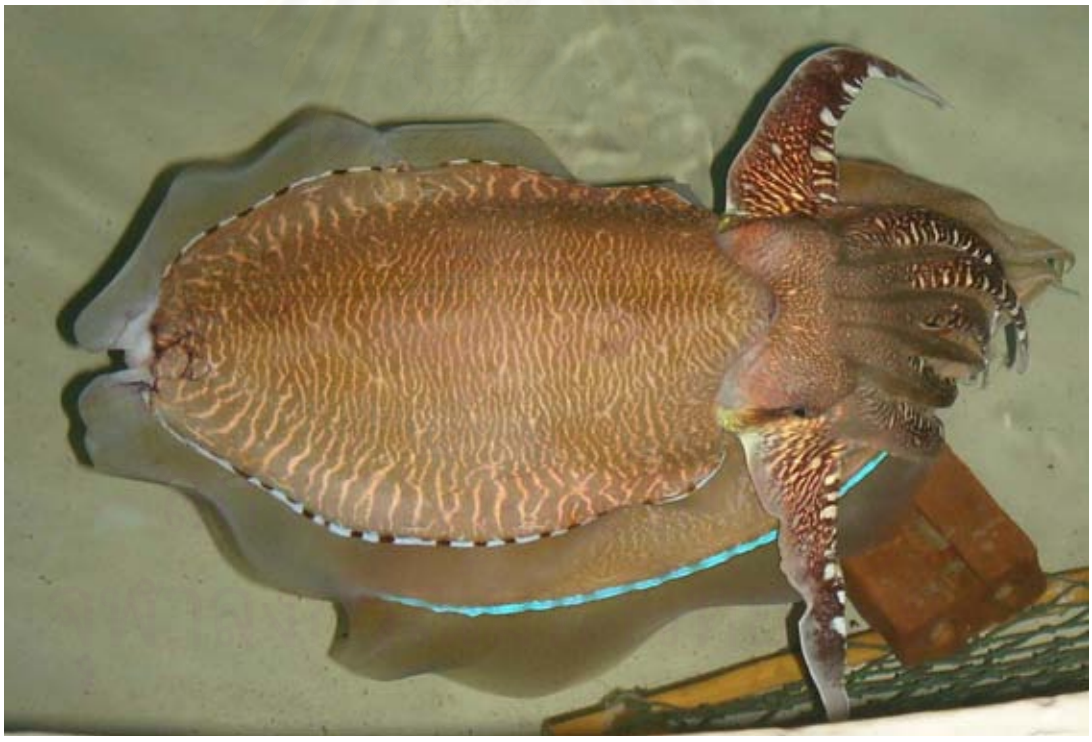


Fig. 3.29 The Buffalo Display in male *S. pharaonis*.

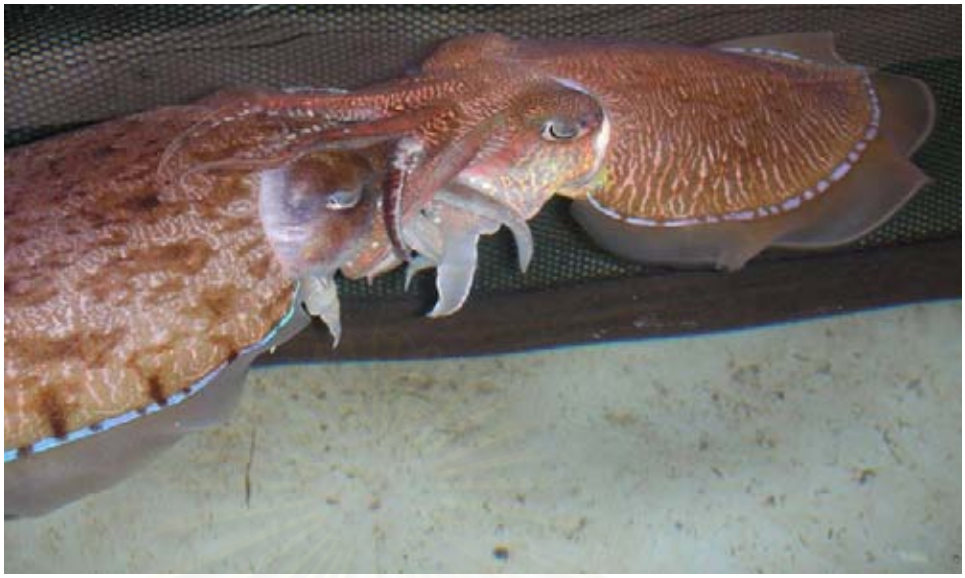


Fig. 3.30 Copulation of *S. pharaonis* in the head-to-head position, with the female on the left, and the male on the right.

In an unsuccessful initiation of copulation, the female may have jetted away as soon as the male approached with the Round Face Tiger Display, or she may have escaped during his attempt to touch and grasp her from above. After an unsuccessful attempt, the male typically tried again repeatedly. In some cases, the female may not have resisted much, therefore the male only had to re-grasp her arms a few times before he finally succeeded. In the cases that the female did put up a heavy resistance, the male may have tried again over and over, but after a series of repeated failures, he often retreated to a distance, and put on another distinct display, called in this study, the Intermediate Display (Table 3.9 and Fig. 3.31). In the Intermediate Display, the male retracted all his arms backward except the third and the fourth arms that were on the side facing the female. The colors of the face area and the retracted arms were pale, while the unilaterally extended third and fourth arms appeared dark. On the body, the contrast level of the tiger stripes decreased, overall color became pale, and a white band appeared on the mid section of the back facing toward the female (unilateral). The edge of the fin became dark, again only on the side facing the female.

Table 3.9 Signal components of the Intermediate Display in *S. pharaonis*

Type of signal components	Descriptions
Arm posture	Arms retracted, pointing backward, except unilateral 3rd and 4th arms on the side facing the female which are extended, and pointing downward with curled tips.
Color pattern on the body	Pale tiger stripes on orange background, low contrast, unilateral white band on mid-body.
Color pattern on the head	Forehead pale, cheek pale, no dark eye rings.
Color pattern on the arms	Pale except the unilaterally extended 3rd and 4th arms which appear dark.
Fins	Dark edge, unilateral.
Mantle edge	White.
Orientation / Movements and other behaviors	Remains still at a distance (2-4 body lengths) from the female. Approaches slowly from time to time.
Skin texture	Smooth.

The Intermediate Display, could be interpreted as a courtship display that signaled an intermediate level of sexual intention, which lied between “very interested to mate” (signaled by the Round Face Tiger Display) and “not at all interested”. The male remained at a distance from the female (about 2-4 body lengths), while showing his Intermediate Display to her. After a period of several minutes, he may have put on the Round Face Tiger Display and tried to approach her again. The new attempt may have been a success, or the male may have been rejected again, and retreated back to his Intermediate Display, awaiting the next chance to re-approach. The oscillation between approaching and retreating may have occurred many times over, until a successful copulation finally ensued, or until the male finally gave up the attempt to mate.

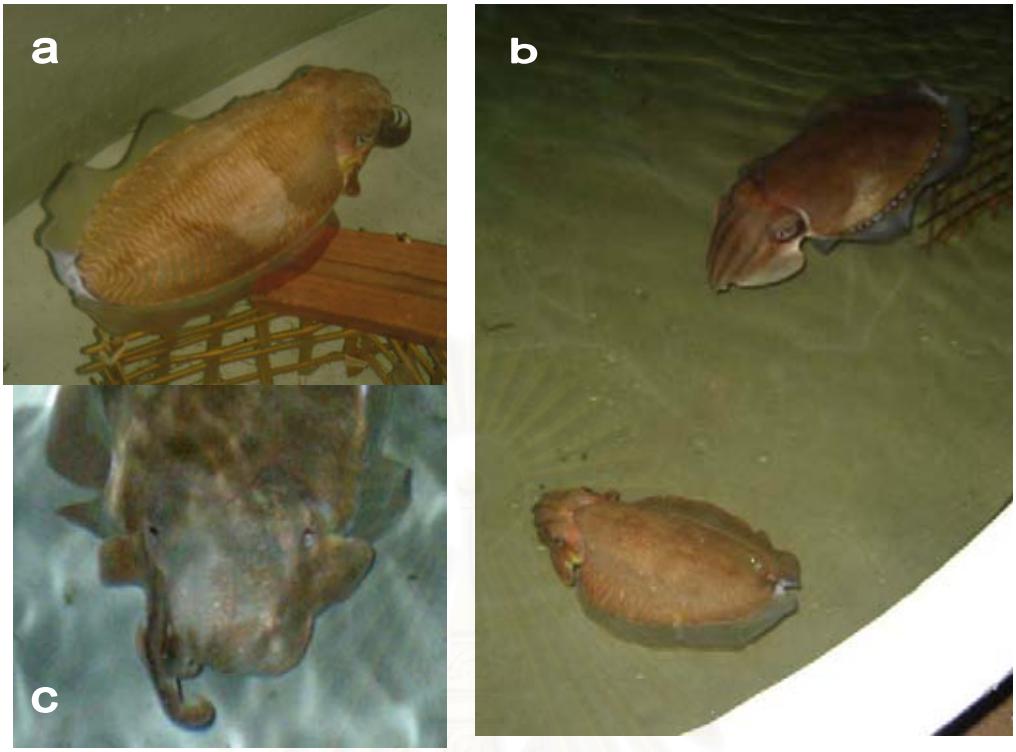


Fig. 3.31 The Intermediate Display, exhibited by male *S. pharaonis* when gets rejected. a) and b) Color pale, white band appears across mid-body, fin edge turns dark only on the side facing the female. c) Arms retracted, except the 3rd and the 4th arms on the side facing the female.

Out of 18 trials in which the first copulation took place (Table 3.10), 8 trials showed the male, almost immediately upon release, rushing to a female and very quickly initiated a successful copulation with her (average time taken to establish copulation = 1 min 30 s, n=8). One trial showed the male, also rushing to a female upon release, but took much longer to establish copulation (20 min), and only did so after many failed attempts. Three trials showed the male approaching both females in turns, and after switching back and forth a few times, finally copulated with one of them (average time to establish copulation = 3 min 30 s, n=3). Five trials showed the male also switching back and forth between the 2 females, but for much longer, and with many more switches, before he finally mated with one of them (average time to establish copulation = 28 min, n=5). One trial showed the male pursuing one female for 10 min, then switching once to the other female, and after pursuing her for 4 min, finally

established a successful copulation. Switching by the male observed during these trials generally occurred in response to rejection by the target female.

In the 7 trials in which mating did not occur within the time limit, the male was either not very active, and did not make a serious effort to mate, or the male was very active but the female managed to jet away every time the male tried to grasp her. In most cases, the male was observed spending most of his time in the Intermediate Display, directing at each female in turns, while making few or no real attempt at copulation (he was either doing this from the start, or after having been repeatedly rejected by both females).

Table 3.10 Different categories of pre-copulatory behaviors observed in trials that mating occurred at least once

Male behavior	Numbers of cases	Average time until copulation (min)
Male very active, mated almost right away, no switch.	8	1.5
Male not as active, approached right away, no switch, but took longer to establish copulation.	1	20
Male switched back and forth only a few times, then quickly grasped one of the females.	3	3.5
Male switched back and forth many times, took very long.	5	28
Male only switched once, but took long time to establish copulation.	1	14

3.3.3 Post-copulatory behaviors

After the first copulation was over, the male typically exhibited close mate guarding by remaining immediately above or next to the female (less than one body length). When the female moved, the male followed very closely, with his arms often still in physical contact with her back (Fig. 3.32a). When the couple happened to face the other female who was also swimming around in the tank, the male often put himself between that female and his mate, then showed the agonistic Intense Zebra Display toward the female, as if she were a threat (Fig. 3.32b).

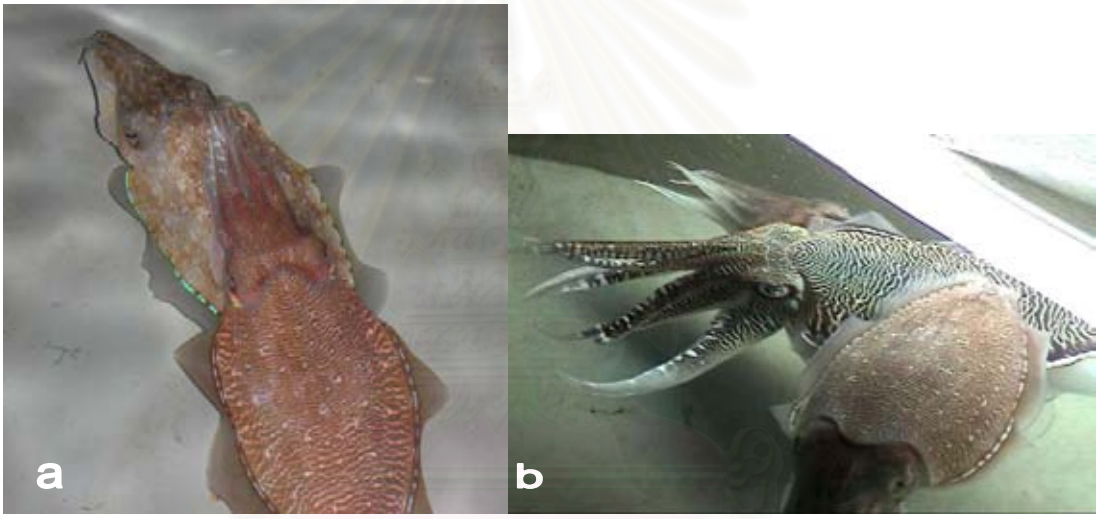


Fig. 3.32 Post-copulatory mate guarding in *S. pharaonis*. a) The male (on top) follows the female around, often resting his arms on her dorsal surface. b) The male shows aggressive Intense Zebra Display toward the other female whom he had not mated with (the female he is guarding is underneath him).

In 8 out of 14 trials in which copulation occurred twice with the same female, the male guarded the female closely after the first copulation and remained on guard continuously until the second copulation took place (Table 3.11). The average interval between the first and the second mating for these 8 trials was 41 min 44 s. In 2 trials, the male guarded the female very closely for a certain period of time (30 min and 1 hour 40 min), and then started switching back and forth between the 2 females for one or more

times, until finally copulated with the same female again. In 4 trials, the male did not guard the female very closely. Soon after the first copulation, the male began to switch back and forth between females a few times, before ended up re-copulating with the same female again. The average interval between the first and the second mating for these 4 trials was 9 min 30 s.

Table 3.11 Different categories of behaviors observed during the period between the first and the second copulation, in trials that re-copulation occurred with the same female.

Guarding beahvior	Number of cases	Average time elapsed until 2nd copulation
Typical close mate guarding all through. No switching.	8	40 min 44 s
Close guarding up until a point, then started switching, but ended up with same female.	2	2 hours 10 m
Did not guard closely. Switched back and forth a few times, then came back to same female.	4	9 min 30 s

In the one trial in which the second copulation was with a different female, the male exhibited close mate guarding for about 7 min after the first mating, then started to switch back and forth, pursuing each female in turns for about 3 min, before finally copulated with the one he had not mated with.

Among the 3 trials in which only 1 copulation occurred, the first one showed the male continuously guarding the female for at least 2 h (after 2 h, the trial was terminated) without ever switching to pursue the other female. Another trial showed the male exhibited typical close mate guarding for an hour, then the female inked profusely, after which point, the male stopped following her, and the trial got terminated. Finally, in the

last one, the male showed typical close mate guarding behavior continuously for 2 h 30 min (no switching), before the trial was terminated. The durations of single copulations that occurred in these trials (15 min, 7 min, and 25 min respectively; mean=15 min 40 s, n=3) were notably longer than usual (compared to a mean of 5 min 38 s from the trials in which re-copulation occurred, n=13), and therefore may have contributed to the reasons why the second copulation did not follow within the time limits of the trials.

CHAPTER IV

DISSCUSIONS

4.1 Sperm removal

This study has confirmed for the first time, the presence of sperm removal behavior in male *S. pharaonis*. This behavior is best explained as a male reproductive strategy arisen under the context of sperm competition to allow self-sperm to have an advantage over their rival during the race for fertilization. In cuttlefish, a large number of sperm is stored inside the seminal receptacles and in the spermatangia attached to the female's buccal membrane. When a female is laying, each unfertilized egg was passed singly through the funnel and then held in a temporary chamber formed by the female's arms, where it is presumably fertilized. In *S. apama*, it has been confirmed that sperm from both the spermatangia and the receptacles are used by the females, and that sperm from the spermatangia are 4 times more likely to be used for fertilization (Nuad et al., 2005). It has also been suggested that the receptacles may function primarily in long term storage, and that sperm from them are only used when spermatangia sperm sources are low (Nuad et al., 2005). Given the case, the act of spermatangia removal by male *S. pharaonis* is likely to have a positive immediate effect on the fertilization success of the remover, and a bias in paternity toward the last male should be expected. Unfortunately, the molecular method selected for the current study has failed to provide sufficient data to support this hypothesis. ISSR-markers chosen were too unreliable, and revealed too little polymorphisms to be useful for the genetic assessment of paternities during this study. These markers may, in the future, be made more reliable, if their PCR-conditions are optimized more thoroughly, and they may become useful for other studies that aim toward studying genetic diversity of *S. pharaonis* at the population level. However, to assess the pattern of sperm precedence and fertilization success associated with sperm removal behavior in *S. pharaonis*, a different approach may need to be taken, namely the development of specific microsatellite markers that

can be used for paternity testing. Such markers have been developed successfully for various other species of cephalopods (*S. officinalis*: Shaw & Pérez-Losada, 2000; *Sepioteuthis australis*: Van Camp et al., 2003; *S. apama*: Shaw, 2003; *S. esculenta*: Zheng, 2007). A future work in *S.pharaonis* should follow the guidelines established by these previous studies.

4.2 Mechanism of sperm removal

The mechanism of sperm removal in *S. pharaonis* differs from what have been described for *S. apama* and *S. officinalis*. In these 2 species, repeated flushing of water through the funnel which aims directly at the female's buccal region was reported as the mechanism of removal (Boal, 1997; Hanlon et al., 1999; Hall & Hanlon, 2002; Nuad et al., 2004). Another study done in *S. esculenta* (Wada et al., 2005), however, reported the scraping actions of the third arms as responsible for removal, which is consistent with the current study. In this study, spermatangia were found strongly attached to the female's buccal membrane after mating. Therefore, it would be quite suspicious if the force of flushing water alone could be sufficient for displacing these spermatangia. In addition, the video footage taken revealed that the male did not always aim his siphon at the female's buccal region during the sperm removal phase. Sometimes, pieces of removed spermatangia could be seen falling out even when the male were not at all flushing at the female's buccal area. Based on the current results, and those of Wada et al. (2005), flushing, as a mechanism of sperm removal in *S. apama* and *S. officinalis*, should perhaps be reinvestigated, in order to determine whether it represents a genuine difference among species. In a future study, perhaps the suckers on the third pair of arms could be surgically removed to see its effect on the efficiency of sperm removal.

4.3 Extent of sperm removal

The second male removed, on average, only about 34% (by numbers) of the spermatangia attached on the female's buccal membrane by the first male. This percentage is possibly still an overestimate of the true proportion of spermatozoa that had been eliminated, since the removed spermatangia were often not wholly intact, but yet each was counted as one (e.g. their bases could still be attached to the buccal membrane. Only the distal portions got removed, fell out, and were counted). Studies done in other cuttlefishes also showed similar incomplete or partial sperm removal, but none has presented a quantitative estimate on the average % removed (Hanlon et al., 1999; Wada et al., 2005). In sperm competition, higher removal rate of sperm should lead to an increase in fertilization success of the remover. If this is the case, then why do male cuttlefish not remove all the previous male's spermatangia when they have the ability to do so?

Partial sperm removal also occurs in some species of Odonata (Siva-Jothy & Tsubaki, 1989). In these cases, the incompleteness of removal did not matter, and the last male still achieved complete sperm precedence, because they selectively displaced rival sperm from only a particular region, from which the female primarily utilized sperm for fertilization. Wada et al. (2005) who studied *S. esculenta* argued that in cuttlefish, there may indeed be a locational priority for fertilization among the spermatangia attached on the female's buccal membrane, but that is unlikely to be the reason for the observed partial removal, because the male's arms are probably too large for the task of selectively removing particular spermatangia from a particular region within the densely packed cluster on the female's buccal membrane. In the current study, casual observation during examination of the female's buccal membrane after the last mating showed that spermatangia deposited near the openings of seminal receptacles were typically the ones from the second mating, while the ones from the first mating that survived removal were often clustered together on the more medial, more toward the inside, region of the ventral buccal membrane. This suggests that the

possibility of selective removal based on the most effective location may not be immediately dismissible in *S. pharaonis*, and further studies on this particular aspect should be undertaken.

Wada et al. (2005) continued to argue that repeated matings, or re-copulation, may compensate for partial sperm removal, such was the case in earwigs *E. plebeja*, whose males displaced only 20% of the existing sperm mass (Kamimura, 2000), but repeatedly mated with the same female and still gained high reproductive success at the end (Kamimura, 2005; Wada et al., 2005). This was, however, still an unlikely explanation for *S. esculenta*, since multiple matings with the same female were rarely observed during a pairing (Wada et al., 2005). For *S. pharaonis* , on the other hand, repeated matings have been shown in experiment III of this study, and therefore may provide a possible reason why complete sperm removal is not needed.

Another mechanism that may compensate for partial sperm removal is called the "last in, first out" principle, which has been demonstrated in many groups of animals such as birds and insects (Birkhead & Møller, 1998; Simmons, 2001). According to this principle, the last sperm to enter the storage organ may push those from previous males to the back and are likely to be the first ones used during fertilizations of the eggs. By this, the last male may still achieve a high level of sperm precedence, even though it only removes a small portion of the first male's sperm. In the current study, the second male's spermatangia were often deposited on top of the first male's spermatangia on the female buccal membrane. It is possible that these uppermost spermatangia will be the ones to come into contact with the unfertilized eggs first when the female starts spawning. The "last in, first out" principle may operate in *S. pharaonis*, and should be subjected to further investigation.

Wada et al. (2005) suggested the most likely explanation for partial removal in *S. esculenta* to be the restriction of time. In *S. esculenta*, mating was often interrupted by another male, therefore, if the mating male had spent too much time trying to achieve a

complete removal, he would probably get interrupted during that period, and would not have a chance to transfer even a single spermatophore. Thus, partial removal could be viewed as a compromise between trying to remove as much rival sperm as possible and trying to transfer spermatophores in time before getting interrupted. For *S. pharaonis*, in order to support this hypothesis, field observations should be conducted to determine how frequent interruptions actually occur in the wild.

Another alternative hypothesis is that the male may not have an ability to perform a complete removal in the first place. There may be a physical constraint that prevents the male from completely removing all of the rival sperm. This is the case for the earwigs *E. plebeja*, in which the male's penis is not long enough to reach into the deeper portion of the female's spermatheca (Kamimura, 2005). For *S. pharaonis*, the physical constraint is unlikely to be the case, since an occurrence of close to 100% removal has been observed during this study. In addition, deposited spermatangia are stored externally on the female's buccal membrane, a site which the male should have little difficulty in gaining an access to during copulation.

In conclusion, the explanation for partial sperm removal in *S. pharaonis* still remains unclear. Further research is needed to investigate whether or not fertilization success parallels the extent of sperm removal. In addition, possible removal of sperm stored inside the seminal receptacles has been largely ignored in this study, and should be investigated in the future.

4.4 Factors related to variations in the amount of sperm removed and the duration of the removal phase

The body size of the mating male, and the size of its Needham's organ did not have a significant effect on the amount of spermatangia that he removed. This finding is consistent with what has been found in *S. esculenta* (Wada et al., 2005). Wada et al. (2005) did not find any significant correlation between the amount of sperm removed during the current mating and all the chosen indicators of the amount of sperm deposited during previous mating (e.g. duration of spermatangia placement during the first mating, number of matings of paired female during the previous 3 and 6 hours). On the contrary, in this study, the strongest factor determining the number of spermatangia removed seemed to be the number of spermatangia that had been deposited on the female's buccal membrane during the first mating (Fig. 3.19a). The second male tended to remove more spermatangia as he found more of them deposited by the first male. However, the proportion or the percentage of first male's spermatangia that was removed did not increase as there were more spermatangia deposited (Fig. 3.19b). This suggests that males may remove a constant proportion regardless of how many spermatangia are present. If this is the case, removing more spermatangia may not automatically lead to greater reproductive success, since it probably depends more on the proportion that is removed, and if the proportion is constant, then a male that is observed to remove only a few spermatangia may have the same level of success as a male who is observed to remove a great number of spermatngia. A future study should perhaps consider this point before attempting to use the absolute amount of spermatangia removed as a predictor for the pattern of paternities that follows.

The number of spermatangia removed was also positively correlated with the amount of time the mating male spent in the removal phase (Fig. 3.20). The same relationship was also found in *S. esculenta* (Wada et al., 2005). However, this study did not find a significant correlation between the percentage of first male's spermatangia removed and the amount of time that the second male spent in the removal phase. This

suggests that the duration of the removal phase may not reflect its actual effectiveness. A male that encounters many deposited spermatangia may spend a long period to remove them, yet in the end may achieve the same proportion of removal as a male who encounters only a few deposited spermatangia and spent only a little time removing them. If this is the case, then any future study should consider this problem before using the duration of the removal phase as an indicator of the extent of removal, or as a predictor of reproductive success.

Nuad et al. (2004) analyzed in *S. apama*, the fertilization success associated with different durations of time spent in flushing, which was thought to be the mechanism for sperm removal in the species. The result showed that longer flushing did not result in more fertilizations by the flushing male. Based on the results of the current study, this needs not be interpreted as ineffectiveness of sperm removal behavior. Rather, as suggested above, the duration of the removal phase (in this case, the flushing time) may not be a reliable indicator of the proportion of spermatngia that are actually removed, and therefore should not be expected to correlate with subsequent fertilization success.

In this study, the first males were found to spend a substantial amount of time manipulating the female's buccal area before inseminating her with their sperm. During the first matings, there were no spermatangia previously deposited, and so there were none to be removed. Yet, the average duration that the first males spent during this "pre-insemination phase" (about 2.5 min) was significantly longer than the average duration that the second males spent during the removal phase (about 1.5 min), when there were actual spermatangia to be removed. This result could represent an artifact, since before the second matings, the females had just come out of anesthetics. In the first matings, compared to the second matings, females may have been more active, and put up more struggles to the male's attempt to copulate, hence the longer pre-insemination time observed may represent the greater difficulty that the first male faced in trying to force the female to remain still. On the other hand, if this was not an artifact, then it could

mean that when there is no spermatangia to be removed, the male of *S. pharaonis* actually spends longer period of time in the removal phase than when there are previously deposited spermatangia present. Perhaps, at the beginning of copulation, the first male first used his arms to probe and try to locate the existing spermatangia deposited on the female's buccal membrane. When he could not find any, he just kept on searching until he was certain that there was none, a process which could take quite some time. In the case of the second mating, the male may have started copulating and found the deposited spermatangia right away. Hence, the searching time was shortened, and this may explain why the second male spent shorter time in the pre-insemination period (the removal phase) than did the first male. If this is true, then in future studies, mating history of the female (whether or not she already has spermatangia present on her buccal membrane) should always be taken into account before interpreting the amount of time the male spent in the removal phase while mating with that female.

The interval between two matings is another factor that might play role in determining the amount of spermatangia removed. The longer the interval, the harder the cement that helps attach the first male's spermatangia to the buccal membrane may become, and the more difficult they might be for the second male to remove. Hence, a negative correlation between the length of the interval and the amount of spermatangia removed is expected. However, no such correlation was found, and the effect was not supported by the current results. Although, the interval between matings in this study (experiment I) only ranged from about 10 min to about 1 hour, which may not be great enough to generate any significant effect. In the study done in *S. officinalis* (Hanlon et al., 1999), pieces of removed spermatangia were only observed during trials that were conducted immediately after the female had been mated, but were not observed in trials that were conducted 1-2 days afterwards, suggesting that the magnitude of the interval may have to exceed 24 hours before its negative effect on the amount of sperm removed can become apparent.

4.5 The attachment-space hypothesis

Wada et al. (2005) proposed a hypothesis that sperm removal behavior may serve to create attachment space for subsequently transferred spermatophores. If this is correct, then factors that indicate the amount of sperm removed (e.g. duration of removal phase) are expected to vary by the factors that indicate the amount of spermatangia deposited during that mating (e.g. the duration of placement phase). In *S. esculenta*, no significant positive relationship was found between the duration of removal phase and the duration of placement phase during the same mating, therefore the hypothesis that the male removes sperm to ensure attachment space was not supported (Wada et al., 2005). In the current study, significant positive correlations were found between the duration of the second mating's removal phase and various other factors that indicate the amount of sperm transferred during the same mating, such as the duration of placement phase, the number of spermatophore transfers, and the overall duration of copulation. At first, this seems to be in support of the attachment-space hypothesis. However, the same relationships were also found between the same parameters of the first matings, in which there was no sperm removed. If such relationships could be found regardless of the presence of removed sperm, then perhaps the positive correlations found in the second matings should not be interpreted as indicative of the function of sperm removal. Moreover, when direct measurements were analyzed, as supposed to factors that might indirectly indicate the amount of sperm removed and the amount of sperm transferred, there was no significant correlation found. The actual number of spermatangia removed did not vary by the actual number of spermatangia attached during the same mating. In addition, the average number of spermatangia that the male attached (about 60) greatly exceeded the average number of spermatangia that he removed during that mating (about 20). If the space was really limited, then the male should have removed more, to match the number of spermatangia that he later attached. In conclusion, most of the results of this study did not favor the attachment-space hypothesis, and the sperm removal behavior

of male *S. pharaonis* is probably not done to ensure attachment space for subsequently transferred spermatophores.

4.6 Removal of self-sperm

In *S. esculenta*, Wada et al. (2005) found that the male indiscriminately removed its own sperm when mating for the second time with the same female whom he had recently mated. In *S. officinalis*, when the male was guarding his mate, and the experimenter replaced that female with another, the male showed no change in behaviors and continued to guard the new female as if she were his original mate (Boal, 1996). These results together suggest that male cuttlefish may lack the ability to recognize a specific female as his own mate. The inability to recognize its own and its partner's mating history could lead the male to subsequently copulate with the same female and begin removing the deposited sperm without knowing that they are his own. However, the studies mentioned above involved either putting many males and females together in a closely confined space (Wada et al., 2005), or parting of the mated pair by the experimenter (Boal, 1996), both of which probably caused unnatural confusion to the male. The question still remains whether or not the male would still remove his own sperm when given a chance to guard his mate continuously without interruption, and allowed to re-copulate with her again on his own accord. In experiment III of the current study, the male were allowed to mate twice under such conditions. The results showed that the second copulations with the same female were almost always shorter than the first ones, which speculatively, could have been due to the omission of the removal phase to prevent removal of self-sperm. However, the matings were only observed from the top, and it was impossible to confirm whether self-sperm removal was present or not. In each trial of experiment I of the current study, each male was paired once with the same female, and typically, a long uninterrupted copulation was observed between the pair. However, in 3 occasions, accidental partings of the mating pair were observed during mid-copulation due to the female abruptly jetting away and breaking free from the male. Once parted, the male typically approached the female again and re-assumed

the head-to-head position quickly (within a few min). Upon this re-initiation of copulation, the male would proceed immediately to transferring his spermatophores, while skipping the removal phase. Self-sperm removal was never observed during these occasions. This suggests that when separation occurs only briefly, and the female still remains in sight during the separation, the male may still be able to recognize her as his current mate, and avoids self-sperm removal accordingly, while re-copulating with her. Although, based on previous studies (Wada et al., 2005; Boal, 1996), this recognition ability probably does not extend to the specific individual level (e.g. when the pair is completely separated and encounters each other again later, the male probably would not recognize the female as a familiar individual).

4.7 Comparative features of spermatophore transfer and copulatory sequence

The act of spermatophore transfer in *S. pharaonis* is identical to what have been observed in other species such as *S. officinalis*, *S. apama*, and *S. esculenta* (Hanlon et al., 1999; Hall & Hanlon, 2002; Wada et al., 2005). However, *S. pharaonis* differs dramatically from other species in that the male always carries out multiple spermatophore transfers, and always does them in sets of two. In *S. officinalis*, the male always transfers only once during a mating, and the single act of transfer passes as many as around 200 spermatophores simultaneously (Hanlon et al., 1999). In *S. esculenta*, the male also transfers once per each mating, but passes on average only 8 spermatophores during a single transfer (Wada et al., 2005). In *S. apama*, usually there is also only one transfer per mating, but in a minority of cases (19% of successful matings), 2 transfers per mating could also be observed (Hall & Hanlon, 2002). The average number of spermatophores that are transferred during each mating has never been measured directly in *S. apama*. However, females have been sampled randomly on the spawning ground in the wild, and the average number of spermatangia found attached on their buccal area was estimated around 16 $\pm$ 11 (Nuad et al., 2005). In this study, *S. pharaonis* males were found to pass 9 ($\pm$ 2) spermatophores during each transfer, which is most comparable to what was found in *S. esculenta*. However, since

transfers occurred multiple times in *S. pharaonis*, the total number of spermatophores transferred during a single mating added up to 77 (± 28), which is much greater than in *S. esculenta*, but still is not as great as in *S. officinalis*. The current study also showed that most males tended to re-copulate with the same female at least twice, therefore even more spermatophores are expected to be added before the pair finally part. The large number of spermatophores transferred at each mating, as found in *S. officinalis* and also in *S. pharaonis*, may represent yet another male tactic to increase its fertilization success, this time by flooding the buccal membrane and the seminal receptacle with self-sperm, possibly in order to dilute the sperm stored from previous matings (Hanlon et al., 1999), or to compensate for the chance of future removal by other males.

Regardless of the number of total spermatophores transferred, the pattern of copulatory sequence in *S. pharaonis* still differs markedly from those of other species. In *S. officinalis*, *S. apama*, and *S. esculenta*, mating may be more likely to get interrupted, therefore males may have resorted to transferring many spermatophores at one time and do it only a few times to get it done as soon as possible. In *S. pharaonis*, perhaps mating is less likely to be disturbed in the wild due to their less dense distribution, therefore the male can afford to transfer only a few spermatophores at a time and really take time to place them well before transferring again (Hanlon, personal comm.). However, if the densities of population distribution can really have such effects, then one would expect *S. apama*, which has the densest aggregate during spawning season to be the species that transferred the most spermatophores at a time. This does not seem to be the case, given the current data. Also, *in S. officinalis*, males spend a very long time in the flushing phase prior to spermatophore transfer (about 6 min or 63% of mating time, Hanlon et al., 1999), which is not consistent with what they should do if they are truly trying to avoid the failure of not being able to transfer spermatophores in time before getting interrupted by other males. The problem of why different species should have different patterns of copulatory sequences is still difficult to answer at this point, since the data on mating systems and mating behaviors in the wild hardly exists for any

species other than *S. apama*. Future studies should focus on obtaining and comparing different species' mating behaviors under natural conditions in order to determine whether the density of population or the frequency of interruption really are key factors in generating the difference observed. In addition, the volume of sperm contained inside each spermatophore should also be taken into consideration, since different species may be different in this respect, and that greater number of spermatophores transferred may not mean greater quantity of sperm (e.g. some species might have large spermatophores, therefore only need to transfer a few of them).

Finally, in cuttlefish, little is known about the flexibility of mating behaviors in response to social changes. The pattern of copulatory sequence observed in each experiment may be dependent upon the setting of that experiment. In *S. esculenta*, matings were conducted with many males in the same tank (Wada t al., 2005), while in this study, there was only the mating pair present. Thus, if the setting for *S. esculenta* is changed to match that of the current study, perhaps the copulatory sequence exhibited will become more similar as well, with more transfers of spermatophores. The same can be applied to *S. pharaonis*. If there are more disturbances by other males during mating, then fewer spermatophore transfers with more spermatophores in each transfer might be observed.

4.8 Mating duration

The mating duration observed in the present study (about 9.5 min) is comparable to what have been reported in *S. pharaonis* (Nabhitabhata & Nilaphat, 1999). In other species, mating takes on average 2.4 min in *S. apama* (Hall & Hanlon, 2002), 5.5 min in *S. esculenta* (Wada et al., 2005), and 10 min in *S. officinalis* (Hanlon et al., 1999). In comparison among these 4 species, *S. pharaonis*'s along with *S. officinalis*'s mating seem to fall at the far end of the spectrum in terms of duration. Although in studies conducted in the other 2 species, matings were carried out when

there were other males present, and this may have been the cause of the shorter durations observed.

In this study, mating duration depended largely on the number of spermatophore transfers and the number of placement phases that followed. There were also signification correlations between the length of each placement phase, the number of spermatophore transfers, and the overall mating duration. In other words in a longer mating, the male tended to carry out more transfers, and spend longer period of time at placing each set of the spermatophores. The cause of the variation in these factors needs further investigation. Sizes of the mating male and female, and sizes of the mating male's Needham organ did not seem to have any significant influences. However, casual observations showed that the same male's behaviors did vary from trial to trial, and that the male typically mated the longest (with the highest number of transfers) after it has had a long 5-7 days break from its last mating trial. This suggests that in each trial, the copulation duration may depend mainly on the reproductive readiness and the internal motivation of the male at that particular point in time rather than any permanent qualities such as size.

In the context of mating order, the first male transferred spermatophores more times, and copulated for a longer period than the second male (first male average 12 min with 10 transfers; second male average 7.5 min with 8 transfers). This difference is at first surprising, since according to sperm competition theory, it should be the second male who is expected to expend more sperm and more copulation time, in order to out-compete the previous male (Wedell et al., 2002). Instead, the reverse was found here. This can perhaps be explained given that the sperm removal behavior is particularly effective. Because if it is so, then the second male may have already won the sperm competition by using the removal tactic, and therefore has no need to further increase its sperm expenditure. A similar explanation has also been proposed for a crayfish species, in which the male was also found to not increase its copulation duration and sperm expenditure when mating with a mated female (Galeotti et al., 2007).

4.9 Conclusions

The present study provides additional evidence for sperm competition in cuttlefish. Many details of male reproductive behaviors in *S. pharaonis*, such as the presence of sperm removal behavior, multiple spermatophore transfers, re-copulation with the same female, and post-copuatory mate guarding have been revealed in this study, either for the first time, or at the level of details that has never before been presented. These behaviors can be considered as strategies by the male to improve its own fertilization success rate over that of the rival, and are best explained in the context of sperm competition. In this respect, many of the findings in *S. pharaonis* are consistent with what have been shown so far in other species such as *S. apama*, *S. officinalis*, and *S. esculenta* (Hanlon et al., 1999; Hall & Hanlon, 2002; Nuad et al., 2004; Wada et al., 2005). At the same time, there are also some details unique to *S. pharaonis*, which raise questions about what conditions might have caused them to evolve, and why such conditions should differ among different species of cuttlefish. The study also establishes *S. pharaonis* as another viable model system for studying sperm competition, and prompts for further investigations into its other reproductive features, such as the mating system in the wild, and the pattern of sperm precedence.

REFERENCES

Adamo, S. A., and Hanlon, R. T. (1996). Do cuttlefish (Cephalopoda) signal their intentions to conspecifics during agonistic encounters? Animal Behavior 52: 73-81.

Baker, R. R., and Bellis, M. A. (1993). Human sperm competition: Ejaculation manipulation by females and a function for the female orgasm. Animal Behavior 46: 887-909.

Birkhead, T. R., and Møller, A. P. (1998). Sperm competition and sexual selection. Academic Press, London.

Boal, J. G. (1996). Absence of social recognition in laboratory-reared cuttlefish, *Sepia officinalis* L. (Mollusca: Cephalopoda). Animal Behavior 52: 529–537.

Boal, J. G. (1997). Female choice of males in cuttlefish (Mollusca: Cephalopoda). Behaviour 134: 975–988.

Córdoba-Aguilar, A. (2003). Predicting mechanisms of sperm displacement based on genital morphometrics in the Calopterygidae (Odonata). Journal of Insect Behavior 16-1: 153-167.

Córdoba-Aguilar, A., Uhía, E., and Cordero Rivera, A. (2003) Sperm competition in Odonata (Insecta): the evolution of female sperm storage and rivals' sperm displacement. Journal of Zoology 261: 381–398.

Davies, N. B. (1983). Polyandry, cloaca-pecking and sperm competition in dunnocks. Nature 302: 334-336.

Eberhard, W. G. (1985). Sexual selection and animal genitalia. Harvard University Press, Cambridge, Massachusetts.

Gage, M.J. G. (1992) Removal of rival sperm during copulation in a beetle, *Tenebrio molitor*. Animal Behavior 44: 587–589.

Galeotti, P., Pupin, F., Rubolini, D., Sacchini, R., Nardi, P. A., and Fasola, M. (2007). Effects of female mating status on copulation behaviour and sperm expenditure in the freshwater crayfish *Austropotamobius italicus*. Behav. Ecol. Sociobiol. 61: 711–718.

Gallup, G. G., Jr. (2004) Semen displacement as a sperm competition strategy in humans. Evolutionary Psychology 2: 12-23.

Hall, K. C., and Hanlon, R. T. (2002). Principal features of the mating system of a large spawning aggregation of the giant Australian cuttlefish *Sepia apama*. Mar. Biol. 140: 533–545.

Hanlon, R. T., and Messenger, J. B. (1996). Cephalopod behaviour. Cambridge University Press, Cambridge.

Hanlon, R. T., Ament, S. A., and Gabr, H. (1999). Behavioral aspects of sperm competition in cuttlefish, *Sepia officinalis* (Sepioidea: Cephalopoda). Mar. Biol. 134: 719–728.

Harcourt, A. H., and Gardiner, J. (1994). Sexual selection and genital anatomy of male primates. Proceedings: Biological Sciences 255: 47-53.

Haubruge, E., Arnaud, L., Mignon, J., and Gage, M. J. G. (1999). Fertilization by proxy: rival sperm removal and translocation in a beetle. Proc. R. Soc. Lond. B. 266: 1183-1187.

Helversen, D.v., and Helversen, O. v. (1991). Pre-mating sperm removal in the bushcricket *Metaplastes ornatus* Ramme 1931 (Orthoptera, Tettigonoidea, Phaneropteridae). Behav. Ecol. Sociobiol. 28: 391-396.

Jereb, P., and Roper, C. F. E. (2005). Cephalopods of the world: an annotated and illustrated catalogue of cephalopod species known to date (Volume 1: Chambered Nautiluses and Sepioids). Food and Agriculture Organization of the United Nations, Rome.

Kamimura, Y. (2000). Possible removal of rival sperm by the elongated genitalia of the earwig, *Euborellia plebeja*. Zool. Sci. 17: 667–672.

Kamimura, Y. (2005). Last male paternity of *Euborellia plebeja*, an earwig with elongated genitalia and sperm removal behavior. J. Ethol. 23: 35–41.

Leonard, J. L. (2001). Cloaca inspection or pecking in Allen's Hummingbird. Western Birds 32: 91-92.

McCracken, K. G. (2000). The 20-cm Spiny Penis of the Argentine Lake Duck (*Oxyura vittata*). The Auk 117(3): 820–825.

Miller, P. L. (1987). Sperm competition in *Ischnura elegans* (Vander Linden) (Zygoptera: Coenagrionidae). Odonatologica 16: 201–207.

Nabhitabhata, J. (1995). Mass culture of cephalopods in Thailand. World Aquaculture 26: 25–29.

Nabhitabhata, J., and Nilaphat, P. (1999). Life cycle of cultured pharaoh cuttlefish, *Sepia pharaonis* Ehrenberg, 1831. Phuket Marine Biological Center Special Publication 19: 25–40.

Naud, M., Hanlon, R. T., Hall, K. C., Shaw, P. W., and Havenhand, J. N. (2004). Behavioural and genetic assessment of reproductive success in a spawning aggregation of the Australian giant cuttlefish, *Sepia apama*. Animal Behavior 67: 1043–1050.

Naud, M., Shaw, P. W., Hanlon, R. T., and Havenhand, J. N. (2005). Evidence for biased use of sperm sources in wild female giant cuttlefish (*Sepia apama*). Proc. R. Soc. B. 272: 1047–1051.

Ono,. T, Siva-Jothy, M. T., Kato, A. (1989). Removal and subsequent ingestion of rival's semen during copulation in a tree cricket. Physiol. Entomol. 14: 195-202.

Parag, A., Bennett, N. C., Faulkes, C. G., and Bateman, P. W. (2006). Penile morphology of African mole rats (Bathyergidae): structural modification in relation to mode of ovulation and degree of sociality. Journal of Zoology. 270: 323–329.

Parker, G. A. (1970). Sperm competition and its evolutionary consequences in the insects. Biol. Rev. 45: 525–567.

Parker, G.A., and Simmons, L.W., (1991). A model of constant random sperm displacement during mating: evidence from *Scatophaga*. Proceedings of the Royal Society of London Series B 246: 107–115.

Reynolds, J. D., Celada, J. D., Carral, and J. M., Matthews, M. A. (1992). Reproduction of astacid crayfish in captivity—current developments and implications for

culture with special reference to Ireland and Spain. Invertbr. Reprod. Dev. 22: 253–266.

Ryan, J. M. (1991). Morphology of the glans penis in four genera of Molossid bats (Chiroptera: Molossidae). Journal of Mammalogy. 72: 658-668.

Shaw, P. W. (2003). Polymorphic microsatellite DNA markers for the assessment of genetic diversity and paternity testing in the giant cuttlefish, *Sepia apama* (Cephalopoda). Conservation Genetics. 4: 533-535.

Shaw, P. W. and Pérez-Losada, M. (2000). Polymorphic microsatellites in the common cuttlefish *Sepia officinalis* (Cephalopoda). Molecular Ecology 9: 237–244.

Simmons, L. W. (2001). Sperm competition and its evolutionary consequences in the insects. Princeton University Press, Princeton.

Siva-Jothy, M. T. (1987). Variation in copulation duration and the resultant degree of sperm removal in *Orthetrum cancellatum* (L.) (Libellulidae: Odonata). Behav. Ecol. Sociobiol. 20: 147-151.

Siva-Jothy, M. T., Tsubaki, Y. (1989). Variation in copulation duration in *Mnais pruinosa pruinosa* (Calopterygidae; Odonata). I. Alternative mate-securing tactics and sperm precedence. Behav. Ecol. Sociobiol. 24: 39–45.

Stockley, P. (2002). Sperm competition risk and male genital anatomy: comparative evidence for reduced duration of female sexual receptivity in primates with penile spines. Evolutionary Ecology. 16: 123-137.

Tsai, P. S. (2006). Comparative biology of reproduction: Lecture note 25: Molluscan reproduction I[online]. Available from: http://www.colorado.edu/intphys/iphy4480tsai/Lecture25.html [2007, May 10]

Van Camp, L. M., Saint, K. M., Donnellan, S., Havenhand, J.N., and Fairweather, P. G. (2003). Polymorphic microsatellite markers for paternity assessment in southern calamari *Sepioteuthis australis* (Cephalopoda: Loliginidae). Molecular Ecology Notes. 3: 654-655.

Villanelli, F., and Gherardi, F. (1998). Breeding in the crayfish, *Austropotamobius pallipes*: mating patterns, mate choice and intermale competition. Freshw. Biol. 40: 305–315.

Waage, J. K. (1979). Dual function of the damselfly penis: sperm removal and transfer. Science 203: 916–918.

Waage, J. K. (1986). Evidence for sperm displacement ability among Zygoptera (Odonata) and the means for predicting its presence. Biol. J. Lin. Soc. 28: 285–300.

Wada, T., Takegaki, T., Mori, T., and Natsukari, Y. (2005). Sperm displacement behavior of the cuttlefish Sepia esculenta (Cephalopoda: Sepiidae). J. Ethol. 23: 85–92

Young, R. E., Vecchione, M., and Mangold, K. M. (2000) Cephalopod spermatophore terminology[Online]. Tree of Life web project. Available from: http://tolweb.org/accessory/Cephalopod_Spermatophore_Terminology?acc_id=1972 [2007, May 10]

Zheng, X., Ikeda, M., Barinova, A., and Taniguchi, N. (2007) Isolation and characterization of microsatellite DNA loci from the golden cuttlefish, *Sepia esculenta* Hoyle (Cephalopoda). Molecular Ecology Notes. 7: 40-42.

APPENDICES

Appendix A

Table A.1 Names of ISSR primers used during screening and their 5' to 3' oligonucleotide sequences.

No.	Primers	Oligonucleotide Sequence 5' to 3'
1	T8701	$(CT)_8RA$
2	T8702	$(AG)_7YC$
3	T8703	$(GT)_6YR$
4	T8704	$(GT)_6AY$
5	T8705	CAA $(GA)_5$
6	T8706	GGGC $(GA)_8$
7	T8707	$(GAG)_4RC$
8	T8708	$(GA)_7RG$
9	T8709	$(GT)_7YG$
10	T8710	$(CA)_7YC$
11	T8711	$(CA)_7YG$
12	T8712	$(GA)_8AT$
13	T8713	$(CT)_8G$
14	T8714	$(GT)_6RG$
15	T8715	$(GA)_6C$
16	T8716	$(CA)_6C$
17	T8717	$(CA)_6T$
18	T8718	$(GA)_6T$
19	TL01	$(CAG)_5$
20	TL02	$(CAA)_5$
21	TL03	$(GACA)_4$
22	TL04	$(GATA)_4$
23	17898A	$(CA)_6AC$

Appendix A (cont.)

No.	Primers	Oligonucleotide Sequence 5' to 3'
24	17899A	$(CA)_6AG$
25	17898B	$(CA)_6GT$
26	SAS1	$(GTG)_4C$
27	SAS3	$(GAG)_4C$
28	HB13	$(GAG)_3GC$
29	HB14	$(CTC)_3GC$
30	HB15	$(GAG)_3GC$
31	UBC809	$(AG)_8G$
32	UBC811	$(GA)_8C$
33	UBC812	$(GA)_8A$
34	UBC813	$(CT)_8T$
35	UBC814	$(CT)_8A$
36	UBC818	$(CA)_8G$
37	UBC824	$(CT)_8G$
38	UBC826	$(AC)_8C$
39	UBC827	$(AC)_8G$
40	UBC840	$(GA)_8YT$
41	UBC841	$(GA)_8YC$
42	UBC845	$(CT)_8RG$
43	UBC848	$(CA)_8RG$
44	UBC857	$(AC)_8YC$
45	UBC868	$(GAA)_6$
46	814	$(CT)_8TG$
47	844A	$(CT)_8AC$
48	844B	$(CT)_8GC$

Appendix B

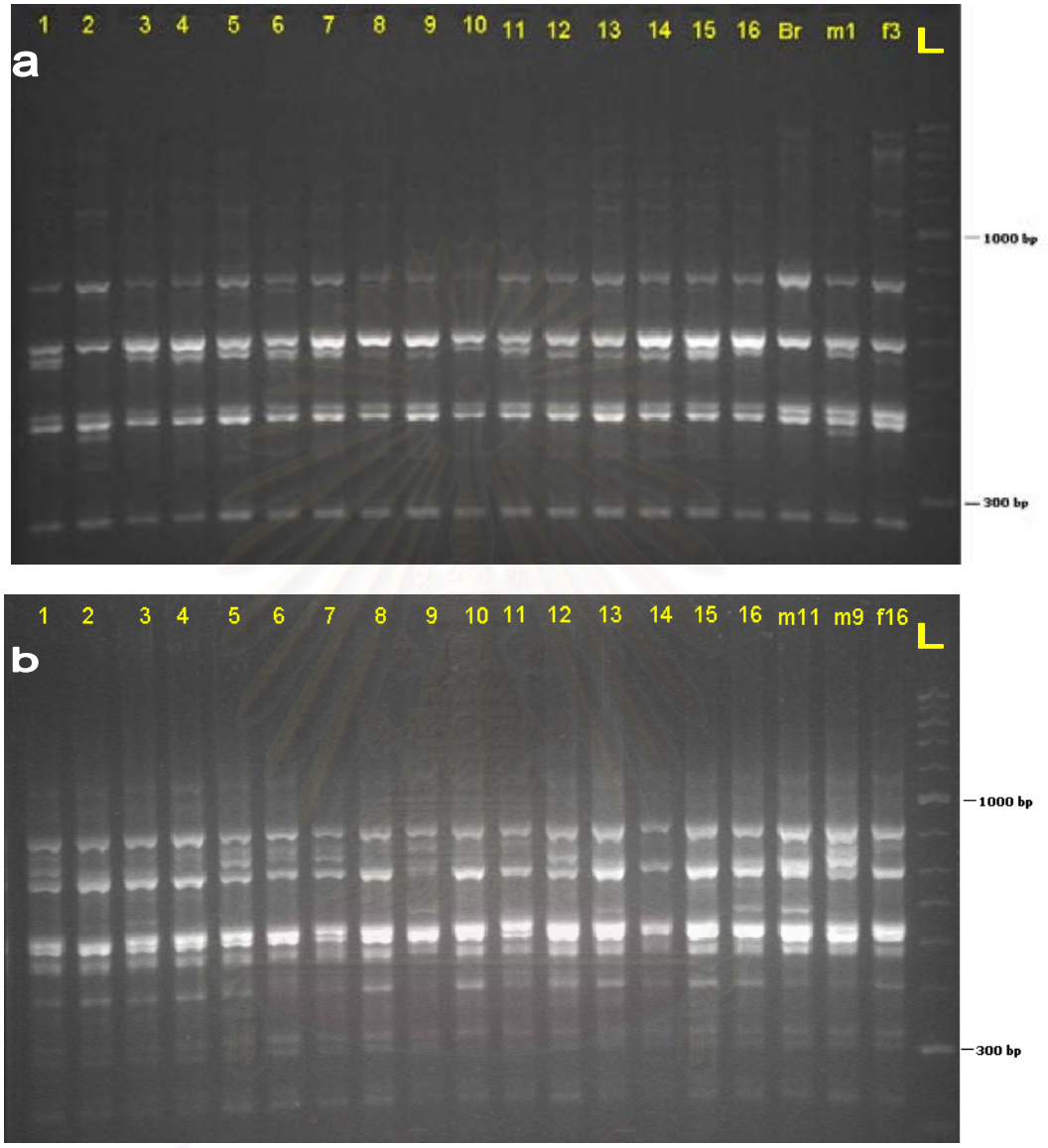


Fig B.1 Examples of ISSR profiles of *S. pharaonis*, run on 2% agarose gel, and visualized under UV light. In a) the marker used was 814, $(CT)_8TG$. Lane f3 indicates the female. Lane Br and m1 indicate the 2 potential fathers. Lane numbers 1-16 represent the sampled offspring from the 1st clutch of eggs that resulted after the mating trial between the males and the female (1st male: Br, 2nd male: m1). In b) The marker used was UBC824, $(CT)_8G$. Lane f16 indicates the female. Lane m11 and m9 indicate the 2 potential fathers. Lane numbers 1-16 represent the sampled offspring from the 1st clutch of eggs that resulted after the mating trial between the males and the female (1st male: m11, 2nd male: m9). In a) and b) lane L represents the 100-bp DNA ladder.

BIOGRAPHY

Tanthai Prasertkul was born on 16 July 1979 in the province of Payao, Thailand. He later moved to Bangkok and completed high school at Samsen Wittayalai. There, he became a student under the Development and Promotion of Science and Technology Talents Project (DPST), sponsored by the Thai government. This program has led him to receive a scholarship for studying abroad in the United States. At Cornell University, He completed a Bachelor of Science, majoring in Biology with a concentration in Neuroscience and Animal Behavior. He later came back to Thailand to pursue a master degree in Marine Biology at Chulalongkorn University. Those who wish to contact the author can reach him via email at yeebud@gmail.com.