Reproductive Biology of Female Swallowtail Butterflies Pertaining to the Pre- and Post-Copulatory Mate Choice

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ABSTRACT

One of the important ways for a female to obtain high reproductive success is to increase the survivability and/or reproduction of her offspring. Then, the quality of sperm used for fertilizing eggs must be critical for female perspective. In swallowtail butterflies, because females have a specially adapted sperm storage organ for fertilization, spermatheca, and because female multiple mating is common, sperm from different males could have a chance to coexist within a female. To increase the reproductive success, multiple mated females should choose sperm derived from different males for fertilization. This is a so-called cryptic female choice. To clarify the mechanisms of cryptic female choice in a polyandrous swallowtail butterfly, *Papilio xuthus*, sperm storage process and the paternity of twice-mated females were examined. Re-mated females ejected first male's sperm from the spermatheca when the second male transferred a lager spermatophore than that of first male, resulting in the biased paternity for the male that had transferred a larger spermatophore. In addition, the significant effect of male dorsal hindwing coloration on their mating success was demonstrated in a wild population of monandrous swallowtail butterfly, *Battus philenor*, in which the color of male dorsal hindwing significantly predicted the spermatophore mass. Since male wing color does not affect competitiveness among males, correlation between male wing color and mating success must be caused by the female choice. Therefore, females in both polyandrous and monandrous species might exercise choice for obtaining sperm of males that can produce a large spermatophore, though the timing of female choice was different between species.

Keywords: cryptic female choice; P_2 value; pre-copulatory mate choice; simplex; spermatophore; sperm ejection; swallowtail butterflies; wing coloration

GENERAL INTRODUCTION

Sexual selection is an evolutionary process that arises from individual variation of fitness, mainly due to the reproductive success rather than its survival (Andersson 1994). A traditional example is the evolution of the long tail in the male guppy, which seemed to be a disadvantage in survival because of the unskillful for swimming to escape from their predators. However, since females prefer to mate with males with longer and more brilliant tail, males with such a long tail must take high mating success resulting in production of many offspring (Bischoff et al. 1985). Recently, it has been widely accepted that the sexual selection plays an important role in shaping traits including secondary sexual traits, sexual dimorphism, reproductive behavior and so on (e.g. Simmons 2001).

Males and females have different behavioral role in sexual selection, due to the limiting factor of reproductive success in respective sex. Bateman (1948) reported a strong relationship between mating and the reproductive success in male fruit flies comparing with females, because of the difference in energetic investment of each sex for their gametes. A single spermatozoon is less costly to be produced than a single ova. Then, a male can produce vastly more gametes than the female, and accordingly, a male can potentially fertilize all the eggs from many females. Therefore, reproductive success of a male is limited by the number of females he can successfully mate with. The ratio of males that are ready to mate to available females to mate (the operational sex ratio) is assumed to be male-biased (Trivers 1972). These situation let males to compete each other.

On the contrary to male perspective, the relationship between mating and the reproductive success in female is relatively weak due to the limited number of eggs, resulting in little benefit from increase in the number of matings. Instead, one of the most important way for a female to obtain high reproductive success is to increase the quality of each of her offspring for survival. Then, quality of sperm used for fertilization must have great influence on female's reproductive success (e.g. Kirkpatrick 1982). An egg fertilized by sperm transferred from an attractive male must be an expert at reproduction in the next generations (e.g. Iwasa et al. 1991).

In general, females in most species mate multiply throughout their lifespan, and morphology and physiology of sperm storage organs have been so widespread that sperm from different males could frequently coexist within the females (e.g. Parker 1970). Thus, females must have opportunity to choose sperm derived from different males for fertilization. In order to inhibit the automatically mixed sperm in the sperm storage organs, females might have to develop specialized behaviors, features or mechanisms, resulting in the biased paternity for preferred male. This post-copulatory process inducing biased paternity is so-called cryptic female choice (Thornhill 1983). Eberhard (1996) proposed many kinds of potential mechanisms for the cryptic female choice such as premature interruption of copulation, lack of sperm transport to storage, the ejection of sperm in storage, lack of ovulation, selective abortion, and so on, though most of them remained unproved due to the technical difficulty for each species (e.g. Albo et al. 2013).

On the other hand, pre-copulatory mate choice might also increase female reproductive success, as suggested by Andersson (1994). Refusing unpreferred males before copulation might be one of the factors for females to use sperm of good males. However, there seems to be some cost on the pre-copulatory mate choice. For example, female mate refusal sometimes induced a strong harassment by the courting males (Huchard et al. 2012). In addition, for both sexes, pre-copulatory mate choice is often time-consuming, and may increase the risk of predation (Pomiankowski 1987). Therefore, the pre-copulatory mate choice by females have evolved where the benefit outweighes such cost. That is, when females do not choose males after mating, the pre-copulatory mate choice must be only the chance for those females to improve their reproductive success. Accordingly, females in monandrous species are expected to develop mate choice behavior before the mating, rather than that in polyandrous species.

Butterflies are one of the most commonly-used organisms for studying sexual selection. During the mating, a spermatophore that contains various kinds of nutrients is transferred from males to females. Since these nutrients are used for egg production and somatic maintenance (Boggs and Gilbert 1979), the fecundity of mated females positively correlates with spermatophore mass (e.g. Oberhauser 1997; Torres-Vila and Jennions 2005) as well as the number of spermatophores transferred (e.g. Watanabe 1988). On the other hand, the large spermatophore could induce the female refusal behavior and increase the refractory period (Sugawara, 1979), resulting in more eggs being fertilized by the male's sperm. As a consequence, males that can produce a larger spermatophore may have higher reproductive success, and the females might be able to produce sons that have ability to produce a larger spermatophore if they use sperm of such males for fertilization.

It has been recognized that there is excessive sexual dimorphism of wing coloration in butterflies; while males of many species have brilliant colored wings, female are cryptic or mimetic on usual. The selective forces generating this difference have been discussed over a century (Allen et al. 2011), and the major selective force for female cryptic coloration is sex-specific natural selection, such as intense predation on females (Ohsaki 1995). On the other hand, adaptive significance of male-limited brilliant wing coloration have been still ongoing debate, though it has been believed that brilliant color of males evolved in the context of female choice (e.g. Wiklund 2003).

The aim of the present thesis is to investigate the behavioral significance of pre- and post-copulatory female choice on the butterfly sexual selection. Firstly, to clarify the mechanism of cryptic female choice and female preference at post-copulatory, sperm storage process after multiple mating of female and its effect on paternity was examined by using the Asian swallowtail butterfly, *Papilio xuthus* (Chapter 1 and 2). Secondly, the effect of iridescent blue color found on the dorsal hindwing of male pipevine swallowtail butterfly, *Battus philenor*, on their mating success was examined (Chapter 3). Then, the effect of pre- and post- copulatory female choice on male reproductive success was discussed.

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Chapter 1

Pattern of sperm storage and migration in the reproductive tract of the swallowtail butterfly *Papilio xuthus*: cryptic female choice after second mating

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Chapter 2

Effect of sperm ejection by females on male fertilization success in the swallowtail butterfly, *Papilio xuthus* L. (Lepidoptera: Papilionidae)

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Chapter 3

Indicators of recent mating success in the pipevine swallowtail butterfly (*Battus philenor*) and their relationship to male phenotype

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GENERAL DISCUSSION

Paternity pattern of eggs laid by multiple mated females was reported for a number of lepidopteran species, such as *Bicyclus anynana* (Brakefield et al. 2001), *Colias erytheme* (Boggs and Watt 1981), *Danaus plexippus* (Solensky and Oberhauser 2009), *Papilio dardanus* (Clark and Shepard 1962), *Pieris rapae* (Wedell and Cook 1998), *P. napi* (Bissoondath and Wiklund 1997), *Pseudaletia separate* (He et al. 1995), *Utetheisa ornatrix* (LaMunyon and Eisner 1994), and so on. Since high mean P_2 value (i.e. Proportion of eggs sired by the second of two males to mate with the same female) has been observed in those species, last male sperm precedence seemed to be a rule for sperm utilization pattern in Lepidoptera (Drummond 1984), as well as the majority of other insect species (e.g. Birkhead and Møller 1998). On the other hand, studies on the intraspecific variation of paternity have pointed out that, in Lepidoptera, P_2 value sometimes shows bimodal distribution with peaks of 0 and 1 (Simmons 2001). Consequently, while the most females lay eggs fertilized by the last male's sperm, the rest of females lay eggs fertilized by the first male's sperm (e.g. Cook et al. 1997; Solensky and Oberhauser 2009). Therefore, lepidopteran species have two modes of sperm utilization, the first male sperm precedence and the last male sperm precedence. In the present study, P_2 value was higher when the sperm ejection occurred (Chapter 1 and Chapter 2), though the possibility that the number of second male's sperm have some effect on paternity seems to be remained. Since males do not have an opportunity for access directly to rival sperm in the spermatheca, the results indicated that lepidopteran females exercise cryptic sperm choice after multiple mating.

In *P. xuthus*, P_2 value was different between females to which larger spermatophores had been transferred from the second than from the first male, and females to which larger spermatophores had been transferred from the first than the second male (Chapter 2). To evaluate the effect of relative spermatophore size in detail, re-analysis was conducted. P_2 value was higher when the second male transferred a larger spermatophore (U=26.5, p=0.013, Fig. 1). Thus, *P. xuthus* females biased paternity toward males that had transferred a larger spermatophore. The effect of spermatophore size on P_2 value was also observed for *D. plexippus* (Solensky and Oberhauser 2009), *Pieris napi* (Bissoondath and Wiklund 1997), *Plodia interpunctella* (Cook et al. 1997) and *U. ornatrix* (LaMunyon and Eisner 1993). Then, females choose males on the basis of spermatophore size, that transferred must be a good indicator for not only the production ability of the spermatophore but also the condition of the male, such as the mating history and age. Since the spermatophore size is affected by the nutrition accumulated during larval stage (Delisle and Hardy 1997) and foraged throughout adult stage (Watanabe and Hirota 1999; Watanabe and Kamikubo 2005), an egg fertilized by sperm transferred from a male that transferred a large spermatophore must be an expert at surviving. In addition, due to the arrangement of the reproductive organs in male body, males do not alter the size of spermatophore during the mating (Chapter 3), indicating that spermatophore size might act as an honest signal (but see Arnqvist and Nilsson 2000).

Mating success of male *Battus philenor* in relation to their dorsal hindwing coloration and their age must be under pre-copulatory sexual selection (Chapter 3). It has been considered that the brilliant coloration of male dorsal hindwings is a sexual signal (e.g. Rutowski et al. 1989). In captive situations, males that dorsal hindwing was artificially blackened showed a significantly lower mating success than control males, though males did not use the coloration for mate recognition (Rutowski and Rajyaguru 2013). Therefore, male dorsal hindwing coloration has to be used by females for mate choice. Rutowski et al. (2010) reported that during aerial courtship maneuvers, the male positions himself below the female and flies up in front of her repeatedly, clearly displaying his dorsal wing surfaces to her. On the other hand, the reason for old male mating advantage in *B. philenor* is remained unclear, while higher competitive ability of older males have been clarified in other lepidopteran species (Kemp 2002; Karl et al. 2013).

There might be a possibility that *B. philenor* females exercise pre-copulatory mate choice to be offered a larger spermatophore. In this species, one of the color parameter, chroma, of male dorsal hindwing significantly predicted the spermatophore mass in the field males (Rajyaguru et al. 2013). Generally, the size of spermatophore offered affects the number of offspring (e.g Oberhauser 1997). Therefore, females mated with males with particular wing color must be offered a large spermatophore, resulting in high reproductive success.

In the present study, females of both *P. xuthus* and *B. philenor* exercised female choice, while timing of choice was different. *P. xuthus* exercised cryptic female choice, while *B. philenor* exercised pre-copulatory mate choice, probably because the difference of mating system (i.e. degree of polyandry). Polyandrous *P. xuthus* females can exercise cryptic female choice after multiple mating, without incurring various cost associated with pre-copulatory mate choice. On the other hand, monandrous *B. philenor* females cannot exercise mate choice after mating, so that they have to choose males before the mating. While pre-copulatory mate choice would incur such cost for females, and multiple mating provides extensive benefit for lepidopteran females, monandry is still maintained in definite proportions of species (e.g Drummond 1984). Because females in monandrous species do not mate multiply even when experimentally given continuous access to virgin males (Kaitala and Wiklund 1994), the lack of opportunity must not be a cause of monandry. Further studies for clarifying the evolution of monandry must be required to comprehensive understanding for mating system of Lepidoptera.

Either pre- or post-copulatory mate choice has a huge impact on male reproductive success. Since most lepidopteran females have a right to decide to begin copulation (e.g. Obara 1982), unpreferred males have little chance to transfer his sperm to females. In addition, even when copulate was succeeded, sperm of some males do not be used for fertilization at all (Chapter 2). The cue for mate choice was similar between *P. xuthus* and *B. philenor*: spermatophore mass. Furthermore, the effect of spermatophore size on P_2 value in some species (e.g. Bissoondath and Wiklund 1997; Solensky and Oberhauser 2009) suggests that the females prefer the males being able to produce a larger spermatophore. Thus, female preference might be one of general driving forces of evolution for males on large spermatophore size.

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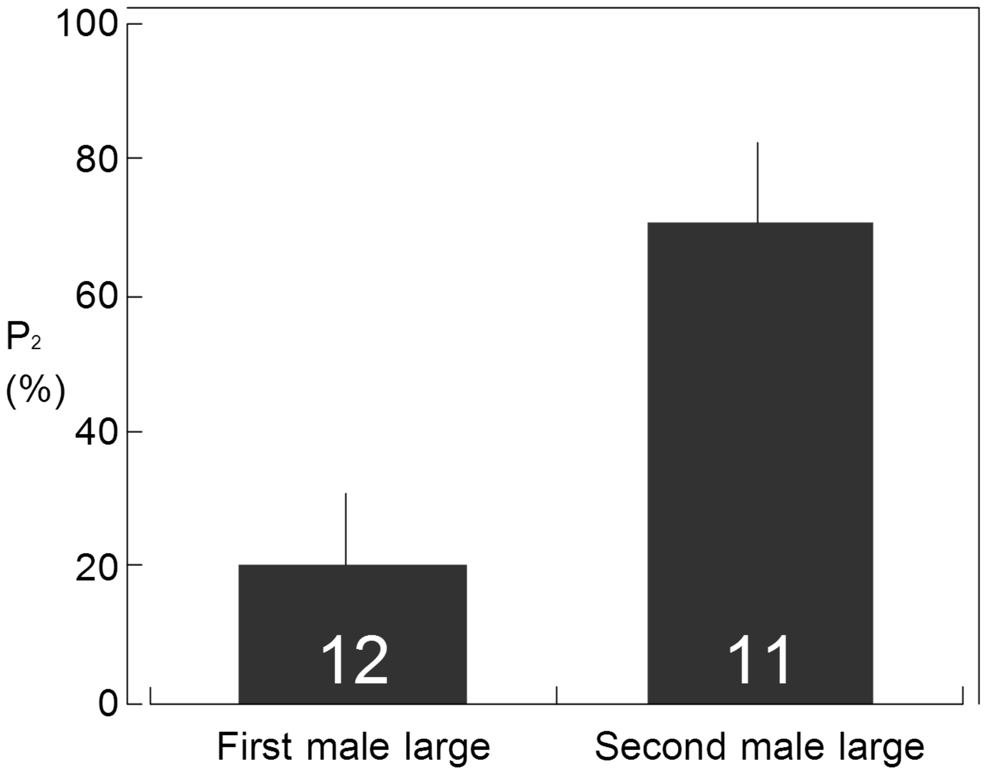

Fig. 1. The P_2 value of females to which larger spermatophores had been transferred from the first than from the second male, and those females to which larger spermatophores had been transferred from the second than the first male (±S.E.).

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