Part 1. Virgin Births Repeated through Generations and in the Individual Females

1.1 Introduction

In the scorpions, the thelytokous parthenogenesis has been made more reliable by the virgin births repeated through the successive generations and in the individual females in two buthids, *Tityus serrulatus* (Matthiesen, 1962, 1971; San Martín and Gambardella, 1966) and *T. bolivianus uruguayensis* (Zolessi, 1985) and an ischnurid, *Liocheles australasiae* (Makioka, 1992, 1993).

Matthiesen (1962) reported that the first generations of *Tityus serrulatus* collected in the field and separately reared in the laboratory gave birth to youngs of the second generation and three second generations became matured and gave birth to the third generations under the separate rearing. A female of the third generation experienced parturitions twice, giving birth to the fourth generations (Matthiesen, 1971). San Martín and Gambardella (1966) also obtained youngs of the third generation in *T. serrulatus* under the separate rearing mostly similar to that in Matthiesen (1962). Zolessi (1985) succeeded in the separate rearing of another *Tityus* species, *T. bolivianus uruguayensis*,

through three successive generations.

These laborious works undoubtedly showed the virgin birth in the two *Tityus* species, but only a small number of the second and third generations obtained left a question whether the virgin birth was an unusual phenomenon. At the same time, another question was also left unsolved whether the virgins reproduced by the thelytokous parthenogenesis or maybe by the self-fertilization in hermaphroditism.

In *Liocheles australasiae*, females of the first generation collected from the field gave birth to youngs of the second generation under the separate rearing in the laboratory and the second generations became matured to give birth to the third generations in the same condition (Makioka, 1993). On the other hand, some females of the first generation repeated pregnancies up to three times and parturitions twice to produce the second generations under the separate rearing (Makioka, 1992). During the repetition of pregnancies in females in the first and second generations, neither sperms nor any other adult male gonadal elements were found in their reproductive systems (Makioka, 1992, 1993). These facts strongly suggested that the thelytokous parthenogenesis is performed as the normal manner of reproduction in the first and second generations, but not yet in the third and the subsequent generations. Furthermore, the processes and mechanisms of the thelytokous parthenogenesis were left still unknown.

A number of virgin females ready to reproduce parthenogenetically are needed for clarifying the cellular processes and mechanisms for the thelytokous parthenogenesis in *Liocheles australasiae*. In Part 1 of the present study, I have separately reared a number of virgin females to make the thelytokous parthenogenesis as the normal manner of reproduction in *L. australasiae* more certain, based on the repetition of the pregnancies through the third and the subsequent generations and in the individual females four times or more. In the course of study, among these virgins, I have obtained many specimens ready to begin the egg maturation and the subsequent embryonic development in their ovaries for the materials of Part 2.

1.2 Materials and Methods

Females of *Liocheles australasiae* (Fabricius) were collected from Iriomote Island, the Ryukyu Islands, Japan, in 1994, as specimens of the first generation. In the laboratory, they were kept separate in glass vials (27 mm in diameter and 55 mm in height) with a piece of wet filter paper, preserved in a dark chamber at 28 ± 1°C, and fed some termites, *Reticulitermes speratus*, once a week.

The first instar juveniles born from their mother immediately climbed up onto the maternal back, stayed there without eating for about a week (Fig. 1A), and then molted into the second instar juveniles (Fig. 1B) to leave their mother and to take food by themselves. Each juvenile was kept separate in a new glass vial soon after the first molt, serially numbered to be distinguished thereafter, and reared as well as the first generations. Through the production of the subsequent generations, the mothers and their offsprings were separately reared under the same condition.

The reproductive systems of the adults and juveniles of late instars were removed from the live or dead specimens in the isotonic physiological saline containing 0.7% NaCl and 0.3% $CaCl_2 \cdot 2H_2O$. In the latter case, the specimens were dissected as soon as possible, mostly within several hours, after the death. They were observed and measured under a

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stereomicroscope, and then fixed with Bouin's solution for the histological study. Juveniles of early instars were directly fixed with Bouin's solution. These fixed specimens were dehydrated in a graded ethanol and *n*-butyl alcohol series, embedded in Paraplast®+ (Oxford Co.), serially sectioned (5 μm thick), stained with Mayer's hematoxylin and eosin, and observed under a light microscope.

1.3 Results

1.3.1 Virgin births repeated through generations

1.3.1.1 Separate rearing from the first
through the fourth generation

Under the separate rearing, adults gave birth to juveniles of the next generation by themselves. Many of the juveniles died in early postembryonic stages and some remnants became matured through five or rarely six molts, about 15 months after the birth, subsequently the new adults became pregnant by themselves, and gave birth to neonates of the new generation about 8 months after the final molt.

Among 147 first generations, only 6 strains were maintained by the virgin births up to the fifth generation. Certainly much more strains attained the second and third generations, but the fourth and fifth generations were difficult to be attained.

For example (Fig. 2), a female of the first generation numbered 94-N54 gave birth to 16 second generations. Only two of the second generations, 94-N54-8 and 94-N54-13, became matured and gave birth to the third generations. Almost all the offsprings of 94-N54-13 died before producing the fourth generations. Only a survivor, 94-N54-13-202, is now

in the seventh instar. Two of the third generations (94-N54-8-2 and 94-N54-8-5) derived from 94-N54-8 became matured and gave birth to the fourth generations.

All the neonates born from 94-N54-8-2 on 20 March 2000, dropped off the maternal body to be eaten by their mother within a few days after the birth. The mother, 94-N54-8-2, is now in the second pregnancy in her glass vial. Another third generation, 94-N54-8-5, gave birth to 21 neonates of the fourth generation on 6 February 1999, as her first parturition. At present, 12 of them are alive, 4 in the fifth, 7 in the sixth, and the remaining one in the seventh instar. The latter 8 are now in the first pregnancy. The mother, 94-N54-8-5, gave birth to 8 neonates of the fourth generation on 9 October 1999, as her second parturition, and died in the third pregnancy on 19 April 2000. Five juveniles of the 8 are now in the fifth instar.

1.3.1.2 Formation of the ovary and female germ cells

The reproductive system is first recogniged as cell masses scattered on the fine lobes of the midgut gland in the third instar juvenile of each generation. These cell masses were distinguished from other tissues in their basophilic stainability and their inclusion of a few oogonia or spermatogonia of about 5 μm in diameter and very early oocytes or

spermatocytes less than about 8 µm in diameter. They were too young for their sexes to be distinguished.

These cell masses gradually extended into fine gonadal cords, which met and fused each other to construct a young gonadal network consisting of three longitudinal and four transverse gonadal cords by the end of the fourth instar (cf. Figs. 3A, 4A). Several oogonia or spermatogonia and very young oocytes or spermatocytes were aggregated along the longitudinal and transverse gonadal cords to form a roughly dotted line of young germaria or germ cell-nests. In the gonad of late fourth instar, there were several oocytes obviously identified by their large size, up to about 30 µm in diameter, surrounded by monolayered follicle epithelia and placed among a number of young oocytes or spermatocytes smaller than about 10 µm in diameter and several oogonia or spermatogonia. The sexes of these younger germ cells could not be still determined.

In the fifth instars, the gonadal cords thickened and became tubular. The gonadal wall, surrounding the gonadal lumen, was thicker in the ventral side, consisting of a monolayered inner and a multilayered outer epithelium, but in the dorsal side, the outer epithelium poorly developed. The largest oocytes near the final size, about 50 µm in diameter, were ready to protrude outward from the ventral wall of the gonadal tube. Each of these oocytes was surrounded by a monolayered follicle epithelium and accompanied by some of the inner and outer epithelia budding from

the gonadal wall.   Late in the fifth instar, the germaria, which had included oogonia or spermatogonia, disappeared, leaving a number of small oocytes or spermatocytes less than about 10 µm in diameter and larger oocytes arranged in a line along the ventromedian axis of the gonadal tube (Fig. 3A, B).

Just after the final molt, the gonad acquired the characteristics of ovary.   The germ cells became clearly observable, because they shifted their position from the gonadal wall to the tips of the short branches of the gonadal tube, i.e., the ovarian diverticula, peculiar to the adult ovary (Fig. 4A, B).   In all adults examined, the gonad included a number of ovarian diverticula of various sizes carrying their own oocytes of the corresponding sizes.   There were practically observed no oocytes remaining in the gonadal wall, implying that the additional supply of the diverticulated oocytes to the ovary does not occur.   Neither sperms nor any other male gonadal elements were found through the gonads.   Hence, the gonads of *Liocheles australasiae*, which prove to contain only female germ cells, are the ovaries, not the hermaphroditic gonads.

1.3.2 Virgin births repeated in the individual females

1.3.2.1 Ovaries and ovarian diverticula in virgins

A total of 147 first generations kept separate in their vials gave birth to neonates of the second generation. Fifty-one second generations became matured, subsequently became pregnant by themselves, and gave birth to neonates of the third generation.

Thirty-three second and third generations became matured and repeated pregnancies and parturitions, but 23 of them died before the third parturition. Eight of the remaining 10 died after the third parturition, one before the fourth pregnancy and 7 in the fourth pregnancy. The other two females experienced the fourth parturition; one died before the fifth pregnancy and another was dissected early in the fifth pregnancy.

The female reproductive system comprised an ovary and a pair of oviducts. The ovary consisted of three longitudinal and four transverse ovarian tubes constructing an ovarian network (Figs. 4A, 5A, 6A). The thick and straight oviducts connected between the frontal ends of the left and right longitudinal ovarian tubes and a genital pore. There were observed no particular structures for preserving sperms, such as spermathecae.

In an ovary in the second pregnancy, for example, a number of ovarian diverticula containing oocytes of various sizes, those containing embryos equal in developmental stage, and those emptied by birth of their embryos at the first parturition ventrally protruded from the ovarian tubes (Fig. 5A, B). The former two types of ovarian diverticula were

larger in proportion to sizes of their oocytes or embryos and the latter

smaller to the days after they had released their embryos. Sometimes

fully grown embryos failed to be born were observed to be retained in the

ovarian diverticula, but they gradually degenerated.

In the ovaries after the final molt and before the first pregnancy,

there were found only the former ovarian diverticula containing oocytes.

In the ovaries in the first pregnancy, there were a number of the former

two types of ovarian diverticula containing oocytes and embryos, but not

the latter.   The ovaries after the first parturition and before the second

pregnancy included the former ones containing oocytes and the large latter

ones, and on the other hand, the ovaries after the second parturition and

before the third pregnancy included the former ones containing oocytes

and the large and small latter ones.   In the ovaries after the third and

the fourth parturitions and before the next pregnancy, there were the

former ones containing oocytes decreasing in number and the small latter

ones increasing in number and decreasing in size corresponding to the

number of times of the parturition (Fig. 6A, B).

1.3.2.2 Histology of the female reproductive systems

Structural features of the adult female reproductive systems were

examined histologically for all the functional phases mentioned above.

The wall of the ovarian tube consisted of two layers of the ovarian epithelia, a multilayered outer epithelium and a monolayered inner epithelium (Figs. 7, 8). The outermost layer of the outer epithelium of the ovarian wall was transformed into a longitudinal muscular layer. The wall of the ovarian diverticula also consisted of the two layers continuing with those of the ovarian wall, but no muscular layer developed in the outer epithelium (Figs. 7, 8). The oviductal wall was extremely thicker than that of the ovarian tube because mostly of a thick muscular layer of the outer epithelium.

Growing oocytes of various sizes (25-50 µm in diameter) were all contained near the tips of the small ovarian diverticula, surrounded by a layer of follicle epithelium (Figs. 7, 8). Fully grown oocytes, mature eggs, and developing embryos were found in the large ovarian diverticula (Figs. 7, 8), the number of which was limited to about 20 per ovary. Neither oocytes nor oogonia remained in the wall of the adult ovarian tube after the final molt, suggesting no new supply of the small ovarian diverticula during the repetition of pregnancies.

Neither sperms nor any other male gonadal elements were found in the female reproductive systems in all the functional phases through the second and third generations examined.

1.4 Discussion

In the present results, the virgin birth was confirmed in *Liocheles australasiae* based upon the separate rearing of a number of specimens through the successive generations. These specimens repeated the life cycles as females from the first through the fourth generation, a few of which became pregnant with embryos of the fifth generation. Furthermore, several specimens in these generations repeated pregnancies at most five and parturitions at most four times. The pregnancies repeated in these specimens must have been carried out without any male participation, because these specimens were completely separated from infancy. Neither sperms nor any other male gonadal elements, such as the mature testicular tissues, as well, were found in the reproductive systems of these specimens through the generations.

Therefore, it was concluded that the specimens became pregnant neither by the bisexual fertilization nor by the self-fertilization in the hermaphroditism, but by the parthenogenesis. Additionally, the embryos developed certainly from the eggs in their ovarian diverticula, denying a little possibility of the asexual reproduction.

At the same time, these results renewed the numbers of successive generations and of the pregnancies and parturitions in the previous studies in *Liocheles australasiae* (Makioka, 1992, 1993) to make the

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parthenogenetic virgin birth as the usual manner of reproduction in *L. australasiae* more certain and more reliable.

Through the separate rearing in Part 1, a number of specimens born obviously by the parthenogenesis, becoming adults, and ready to singly become pregnant were obtained as the materials for the study on the process and mechanism of parthenogenesis in *Liocheles australasiae*. Such a number of specimens for the further studies have never been obtained in the previous studies on parthenogenesis not only in *L. australasiae* (Makioka, 1992, 1993), but also any other scorpion species (Matthiesen, 1962, 1971; San Martín and Gambardella, 1966; Zolessi, 1985).