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Masculinization of female *Eriocheir sinensis* by injecting the extract of androgenic gland of *E. sinensis* and *Scylla paramamosain*

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Abstract: This is the first report of sex reversal induced by injection of androgenic gland (AG) extract in brachyuran crabs. In the present study, the extract of AG from the mud crab *Scylla paramamosain* and the Chinese mitten crab *Eriocheir sinensis* was injected into newly developed female (i.e. just after sex differentiation) crabs *E. sinensis*. Masculinization was observed in the injection with *E. sinensis* as well as *S. paramamosain*. AG cross-activity might exist between the two species. Sex reversal could be achieved at a low dosage of 0.14 (*E. sinensis*) or 0.06 (*S. paramamosain*) AG equivalent.

Key words: *Eriocheir sinensis*; *Scylla paramamosain*; androgenic gland (AG); sex reversal; injection

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Androgenic gland hormone (AGH) in malacostracans is related to sex differentiation and the development of the secondary sexual characteristics of males. Implanting androgenic gland (AG) or injecting androgenic gland extract into juvenile females results in masculinization. Ablation of androgenic gland induces feminization. The masculinization and feminization effects were reported in the beach hopper *Orchestia gamarella*[1], the wood lice *Armadillidium vulgare*[2-5], the crayfish *Procambarus clarkii*[6-8], the freshwater prawn *Macrobrachium rosenbergii*[9-12], and the shrimp *Palaemonetes varians*[13]. During crab culture, there is a significant difference in growth rate between male and female crabs, and sexual maturation in males is earlier than that in females[14]. In mix-sex culture, cannibalism occurs especially among crabs at different molting stages and between the two sexes[15]. So the development of mono-sex culture of crab that could reduce cannibalism and thus enhance survival is of great interest to aquaculturists. Androgenic gland and androgenic gland hormone could provide a reliable method to acquire mono-sex culture.

In crabs, the studies on androgenic gland hormone are especially difficult because of the location and pattern of the cell arrangement of this gland. The androgenic gland is located among the muscles of the coxopodite of the last walking leg attached to the subterminal region of the vas deferens, i.e., the posterior vas deferens[16], and elongates along most of the duct[17-20]. It is situated outside the circular muscle of the posterior vas deferens and bound by a very thin connective tissue membrane[16]. Isolation of androgenic gland in crabs is difficult and

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time consuming. Ablation of this gland in crabs, unlike studies in isopods, amphipods and other decapods, is nearly impossible. There are only some early reports on the roles of AG in crabs. Masculinization effects were reported in *Carcinus maenas*[21] and *Ocypoda platytarsis*[22] after transplantation of AG (*Carcinus maenas*) or injection of the AG extract (*Ocypoda platytarsis*) into the females. Thus the studies of isolation and purification of AGH in crabs lagged far behind as compared to other malacostracan species such as isopods, prawns, and crayfish. To isolate and purify the androgenic gland hormone (AGH), a reliable, relatively easy and convenient bioassay method is essential. The present study aimed to develop a bioassay of AGH in the Chinese mitten crab *Eriocheir sinensis*. *E. sinensis* is widely cultured in most areas of China and resulting in huge economic value. Its artificial breeding and spawning are highly successful. Very young juvenile crabs of *E. sinensis* are readily available for experiment.

It was found that the action of AGH might not be species-specific. Masculinization of the ovary in amphipod *Orchestia* sp. was observed after treatment with plasma of male *Carcinus* sp.[23]. There also exists cross activity between intra- and inter-species in isopod families such as Armadillidiidae, Porcellionidae, and Scyphacidae[24]. Thus it is possible that cross-reactivity of AGH exists between *Eriocheir sinensis* and the mud crab *Scylla paramamosain*. So another purpose of the present study was to demonstrate the cross-activity of AGH between the two species. By injecting AG extract of *S. paramamosain* and *E. sinensis* into juvenile females of *E. sinensis*, cross-activity of AGH was investigated between the two species.

1 Materials and methods

1.1 Isolation and extraction of androgenic gland (AG)

Mature male crabs *S. paramamosain* and *E. sinensis* were collected from local fish market of Hong Kong and Shanghai respectively. Crabs were dissected, and the AGs with vas deferens were isolated and immersed in crustacean physiological saline (modified from Grau and Cooke[25] and Toullec *et al*[26]: 440 mmol·L^{-1} NaCl, 11.3 mmol·L^{-1} KCl, 13.3 mmol·L^{-1} $CaCl_2$, 26.3 mmol·L^{-1} $MgCl_2$, 23.0 mmol·L^{-1} Na_2SO_4, 10 mmol·L^{-1} HEPES, pH adjusted to 7.4, filter (0.2 μm) sterilized). AGs were separated from vas deferens under dissecting microscope. 500 μL of PBS (13.7 mmol·L^{-1} NaCl, 0.27 mmol·L^{-1} KCl, 10.0 mmol·L^{-1} Na_2HPO_4, 1.75 mmol·L^{-1} KH_2PO_4, pH 7.4 within 1 mmol·L^{-1} phenylmethanesulfonyl fluoride (PMSF), 0.2 μm filter sterilized) was added to about 30 to 36 AGs. The AG tissues were homogenized on ice. The homogenate was centrifuged twice for 20 min at 16 000 g and 4 ℃. The supernatant was transferred and stored at 4 ℃ for future use. The protein concentration of the extract was determined by BCA (Sigma) method.

1.2 Female juveniles of *E. sinensis*

The juvenile crabs were bought from a crab hatchery farm in Rudong, Jiangsu province in 2003 and 2004. They were kept in a glass aquarium (90 cm × 45 cm × 60 cm), where the water was 15 cm in depth and there was 5 cm of sand at the bottom of aquarium. The water temperature was kept at about 20 ℃. Photoperiod was not controlled. Crabs were fed twice daily with minced fish meat. The water was exchanged daily.

1.3 Injection experiment in *E. sinensis*

After being cultured for about two weeks, young female crabs with carapace width above 5 mm (5 – 8 mm, at 4^{th} to 5^{th} crab stages) were injected with AG extract of *E. sinensis* or *S. paramamosain*. The volume of injection was 2.0 – 2.2 μL equivalent to 0.14 – 0.16 *E. sinensis* AG or 0.06 – 0.08 *S. paramamosain* AG. The animals in control group were injected with 2 μL of saline. There were 50 animals per treatment. The presence of male gonopods (the external male sexual characteristic, see Plate 2) was observed after each molt. The treated crabs were kept in glass aquarium at water temperature of 20 – 25 ℃ for 40 – 50 days.

At the end of experiment, all the crabs were fixed in buffered-phosphate formalin solution (40 mmol·L^{-1} Na_2HPO_4, 30 mmol·L^{-1} NaH_2PO_4, in 10% formalin solution).

Sections (5 – 6 μm thick) were made by usual paraffin embedding methods and stained with Delafield's hematoxylin and eosin.

2 Results

After 50 days of culture, about 10% crabs were survival (both treatments and control groups, see Table 1). Male gonopods were observed in 3 of the 6 survival crabs injected with AG extract of *E. sinensis* and 2 of the 5 survival crabs treated with AG extract of *S. paramamosain*. No gonopod was observed in the crabs injected with saline. The rate of masculinization or sex reversal was 50% (AG extract of *E. sinensis*) and 40% (AG extract of *S. paramamosain*) of the surviving crabs.

Tab.1　Summary of injection experiment in *Eriocheir sinensis*

injection	no. of original crabs	no. of survival crabs	no. of sex-reversed crabs	no. of molts when sex reversal occurred
AG extract of *Eriocheir sinensis*	50	6 (12%) #	3 (50%) *	2
AG extract of *Scylla paramamosain*	50	5 (10%) #	2 (40%) *	2
saline	50	6 (12%) #	0	–

Notes: # survival rate; * sex reversal rate of survival crabs

The low survival rate in both treatments and control groups may be due to that the juvenile crabs are too fragile and the laboratory conditions are not optimal for their growth.

From former histological observation, gonoduct and gonia could be found in both normal male and female crabs from their third crab stage[27]. The gonoduct was close to the commissure that separated body lumen from surrounding muscle[27]. In the present study, it was difficult to determine gender based on the internal sexual characters. Plate Ⅱ shows the gonopod of a sex reversed male from female. No difference of gonoduct could be found between male and female, nor between the sex-reversed male and female or male. It was noted that the arrangement patterns of internal organs particularly the hepatopancreas as shown in transverse sections were different between juvenile male and female crabs (Plate Ⅲ and Plate Ⅳ). The arrangement patterns of hepatopancreas in sex-reversed male (Plate Ⅴ) were similar to those of a normal male (Plate Ⅳ) at the fifth crab stage, but different from those of a female at the same crab stage (Plate Ⅲ). The differences in arrangement patterns of hepatopancreas between male and female were also accident in the figures presented by Lee *et al*.[27] although the authors did not discuss this in the paper.

3 Discussion

3.1　Bioassay of androgenic gland hormone activity in crabs

Since the identification of androgenic gland in 1954[1], androgenic gland hormone was only isolated and purified from the isopod *Armadillidium vulgare*[3,28-30]. The gene sequence of androgenic gland hormone is known in the isopods *A. vulgare*[30], *Porcellio scaber*, and *P. dilatatus*[31]. The failure to isolate AGH in other malacostracans is due to the lack of bioassay. There are two types of models that have been chosen for the work on AG and AGH: (1) wood lice *Armadillidium vulgare* mainly used by Japanese[32] and French groups[33] and (2) prawn *Macrobrachium rosenbergii* and crayfish *Cherax quadricarinatus* mainly studied by researchers in USA[9] and Israel[34], respectively. In the second model, the female juveniles are injected with AG

extract and their masculinized characters were observed. To isolate and purify the androgenic gland hormone, a reliable, easy and convenient bioassay method is essential. With the relatively long life cycle and unreliable availability of juveniles of decapods, this method is difficult to be used as a bioassay. There is no established model for the study AG and AGH or bioassay for AGH activity in brachyuran crabs.

The present study is the first report on the masculinization of the female *Eriocheir sinensis* by injecting extract of androgenic gland and the possibility of inter-species cross-activity of AGH between *Scylla paramamosain* and *Eriocheir sinensis*. Sex-reversed male was induced by injecting AG extract from either *E. sinensis* or *S. paramamosain* into newly developed female *E. sinensis*.

In the present study, the animals used were newly differentiated females, i. e., they just completed sexual differentiation and their genders could be determined under dissecting microscope. These crabs were at 4^{th} to 5^{th} crab stage.

It is believed that there is a critical period for organgenesis[4]. In *Armadillidium vulgare*, oviduct seems to appear at about 4^{th} instar and after this instar, the androgenic gland hormone has little inhibitory effect on the development of oviduct[35]. After the onset of sexual differentiation in female, gonads retain sexual bipotentiality through several stages of post-embryonic development (instars 5 to 9). Implantation of one AG into a female could induce gonadal masculinization. Sex reversal commonly occurs and functional males develop if females are implanted with one fresh AG at instar 5 or 6. For the crab *Eriocheir japonicus*, sexual differentiation of pleopods appeared at the third[27] to fourth crab[36] stages in males. In the crabs *Callinectes sapidus* and *Rhithropanopeus harrisii*, sexual differentiation can be determined at the second crab stage[37]. Based on these descriptions, the sexual differentiation in crabs appeared to occur before the second crab stage. Compared to the isopod *Armadillidium vulgare*, it seems to be reasonable that gonads in crabs remain sexual bipotentiality through several stages after the second crab stage. These several stages should be the optimal period for AG treatment. Sex reversal and functional males could possibly be observed after AGH treatment in the several stages after the second crab stage.

In crayfish, the genders of very small animals with carapace length (CL) of 5.0 mm can be determined after 1 to 2 molts from hatching. Larvae of this size usually do not survive after implantation of AGs. When animals with CL 7 – 15 mm were used as recipients, masculinization was observed after one year[8]. Injecting homogenate of AGs into small female crayfish (CL 17 – 19 mm) could also induce masculinization[38].

In *Eriocheir japonicus*, implantation of AG in the female crab of the third crab stage resulted in partial or external masculinization[39]. In the present study, the female crabs were at 4^{th} to 5^{th} crab stage, with gonads that may remain sexual bipotentiality. Besides, the external sexual characteristics are more sensible to AGH than internal sexual characteristics[3]. So in some crabs of the present study, external masculinization was observed as early as the first molt after injection (data not shown). In crayfish, the reversed spine also appeared at the next molt after injecting homogenate of AG[38].

3.2 Presence or absence of masculinization of internal sexual characteristics

In the present study, the masculinization was judged by the appearance of external male sexual characteristic i.e. the gonopod. The internal sexual differences such as the orientations and extensions of the gonoducts[27] were not observed even at the sixth crab stage. In the sex reversed female crabs, masculinization in the internal reproductive system could not be confirmed. Yet it was found that the arrangement patterns of the internal organs of the sex reversed females were almost exactly the same as that of the normal males, and were different from those in normal females. It was still difficult to judge the internal masculinization by this observation, so occurrence of masculinization of the reproductive

system could not be ruled out. Lee *et al*.[39] did not either observe internal masculinization; the masculinized female crabs *Eriocheir japonicus* with AG implantation still maintained female genital duct and had no trace of male genital ducts. In the present study, it is necessary to rear the masculinized female crabs for a longer time until their internal sexual characteristics can be observed so that to confirm whether internal masculinization occurred.

3.3 Effective dosage of AG for masculinization

For experiments on sex reversed or masculinized females by implanting AG, one or two AGs were used in the crab *Eriocheir japonicus*[39], the shrimp *Palaemontes varians*[13], the prawn *Macrobrachium rosenbergii*[11,12,9], the crayfish *Procambarus clarkii*[8], and *Armadillidium vulgare*[40]. Besides, injecting AG homogenate equivalent to one AG induced masculinization with the appearance of male gonopore (i.e. genital pore)[41].

A much lower dosage could induce masculinization in other injection experiments. In *Armadillidium vulgare*, injections of 64 U of the AG extract into young females brought about masculinization of external and internal sexual characteristics. Injections of 10 U of the AG extract were hardly effective in inducing masculinization of internal organs of externally masculinized females[3]. A higher dosage is required for musculinzation of the internal sexual characteristics than of the external sexual characteristics.

In the present study, masculinization was induced by injecting AG extract equivalent to 0.14 (*E. sinensis*) or 0.06 (*S. paramamosain*) AG. Masculinization of *Macrobrachium rosenbergii* can be achieved after injecting 0.0013 AG equivalent into female young prawns (Sun, personal communication). This could be indicative of very high activity of AGH in inducing masculinization.

Injecting AG extract of *S. paramamosain* into newly developed female *E. sinensis* also resulted in masculinization (40% of survived crabs). There may exist cross-activity of AGH between the two species *S. paramamosain* and *E. sinensis*. This kind of cross-activity of AGH was reported in some species. Crossed grafting of AG from *Porcellio dilatatus* into *Armadillidium vulgare* showed response, but on the other hand, the grafted AG from *A. vulgare* had no effect on *P. dilatatus*[42]. Further immunohistochemical study was conducted in nine species of Oniscidae isopod. Using *A. vulgare* AGH polyclonal antibody, the positive reaction was observed in AGs of species belonging to the Armadillidiidae, Porcellionidae, and Scyphacidae families[24]. The androgenic gland was thought to have tissue-specific distribution and structural similarity may exist among AGHs of these species. As there is no information about the structure, the composition even the gene sequence of the AGH in *Scylla paramamosain* and *Eriocheir sinensis*, the similarity or identity between the two AGH is unknown.

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注射中华绒螯蟹及锯缘青蟹促雄性腺提取物对中华绒螯蟹雌蟹雄性化的影响

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摘要：该论文首次报道了经由促雄性腺提取物的注射而在蟹类中引起的性逆转现象。此前关于蟹类促雄性腺活性研究的报道极少，而且蟹类的雄性化均是由促雄性腺的移植所产生。本实验中将锯缘青蟹和中华绒螯蟹的促雄性腺提取物分别注射到刚刚完成性别分化的中华绒螯蟹雌性幼蟹体内，此时幼蟹处于 4 至 5 期，壳宽为 5 ~ 8 mm。注射之后，幼蟹经过大约 1 ~ 2 次蜕皮，此时在注射锯缘青蟹以及中华绒螯蟹的促雄性腺提取物的两组实验幼蟹中均能观察到雄性化现象，而注射生理盐水的对照组实验幼蟹中未能观察到相同现象。由此本实验可以证明促雄性腺确实是蟹类的一种雄性激素，注射促雄性腺提取物能引起雌性幼蟹发生性逆转；同时根据锯缘青蟹和中华绒螯蟹的促雄性腺提取物均能引起中华绒螯蟹雌性幼蟹发生性逆转的现象推测，锯缘青蟹和中华绒螯蟹两种间可能存在促雄性腺的交叉活性；不仅如此，性逆转还能在极低的注射剂量下获得，相当于中华绒螯蟹 0.14 促雄性腺当量和锯缘青蟹 0.06 促雄性腺当量。

关键词：中华绒螯蟹；锯缘青蟹；促雄性腺；性逆转；注射

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Plate Ⅰ Sex reversed crab *Eriocheir sinensis* after injecting AG extract into newly developed female crab (3rd to 5th crab stages)

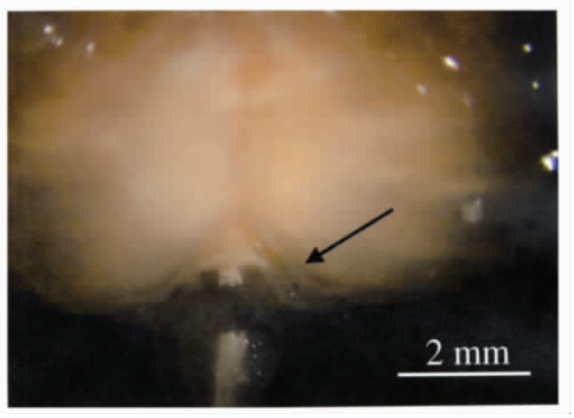

Plate Ⅱ *Eriocheir sinensis* abdomen after injecting AG extract into newly developed female crab (3rd to 5th crab stages) Arrow indicated gonopod that was the external male sexual characteristic

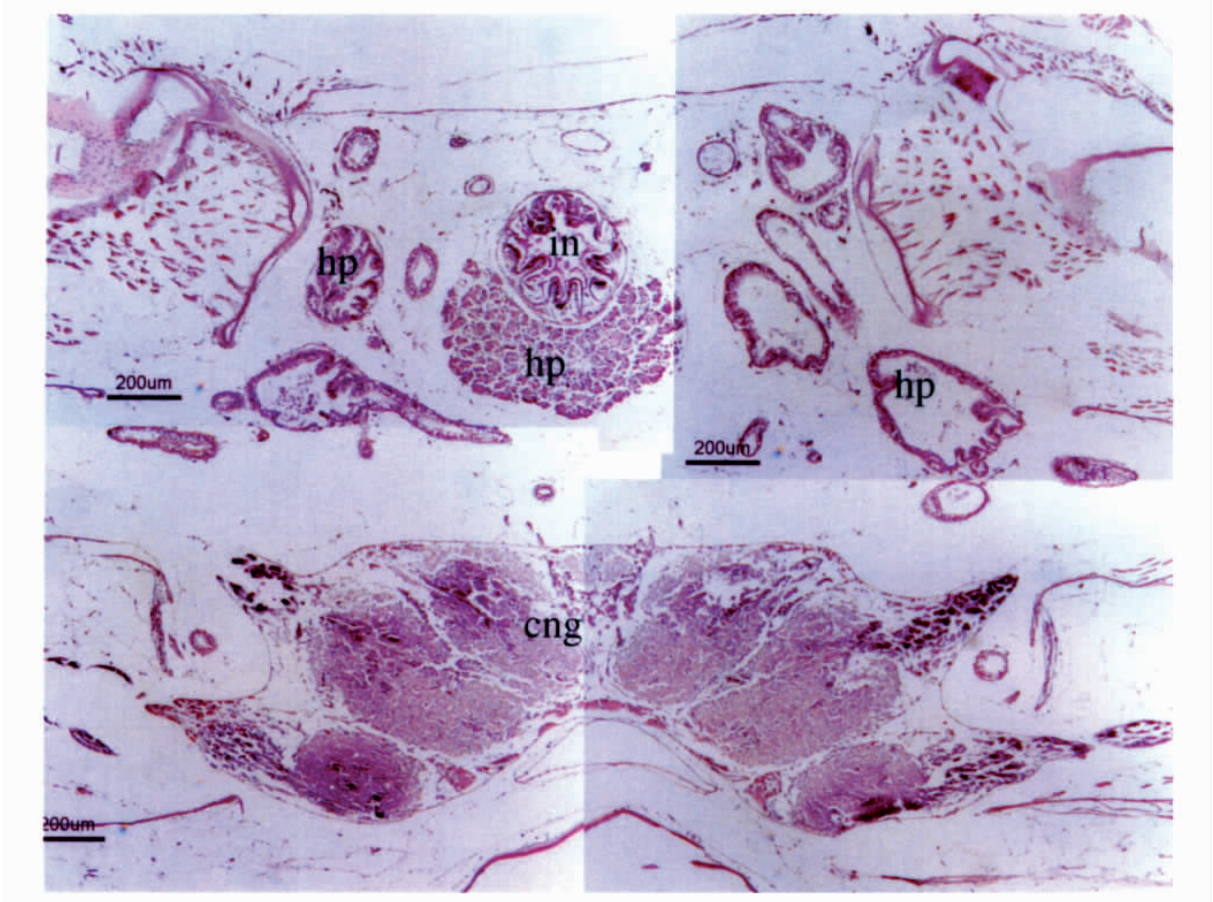

Plate Ⅲ Transverse section of a normal female crab at the 5th crab stage
hp : hepatopancreas ; in : intestine ; cng : central nervous ganglion ; scale bar : 200 μm

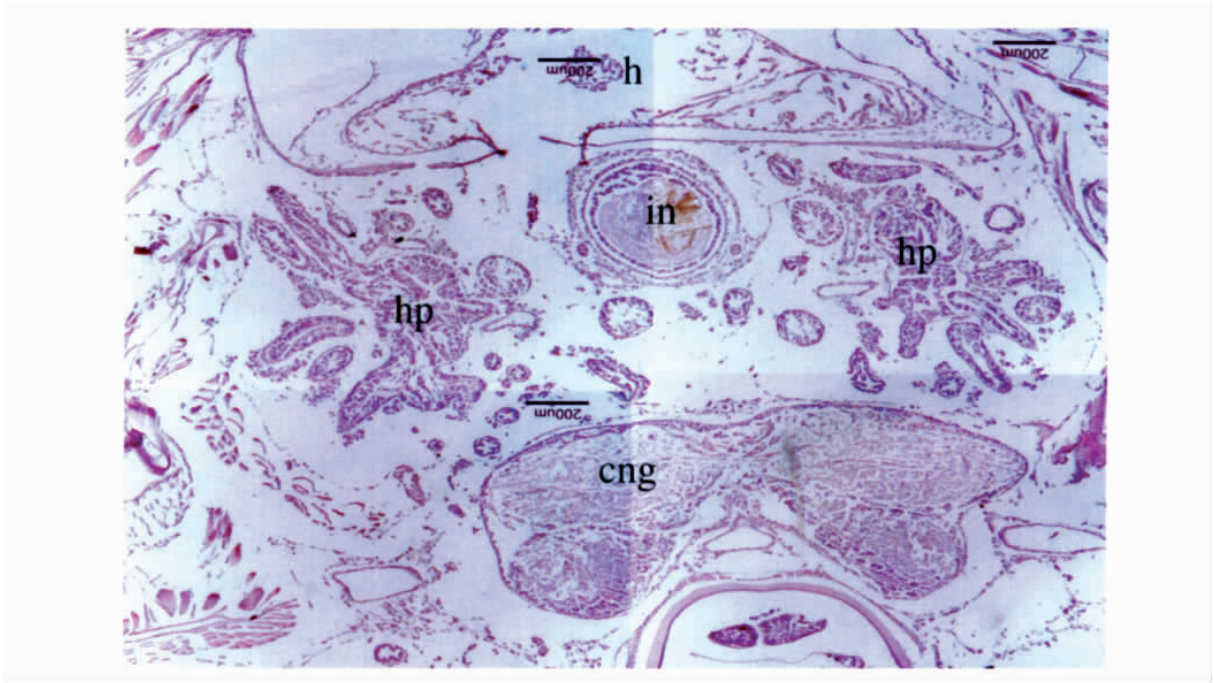

Plate Ⅳ　Transverse section of normal male crab at the 5th crab stage

h：heart；hp：hepatopancreas；in：intestine；cng：central nervous ganglion；scale bar：200 μm

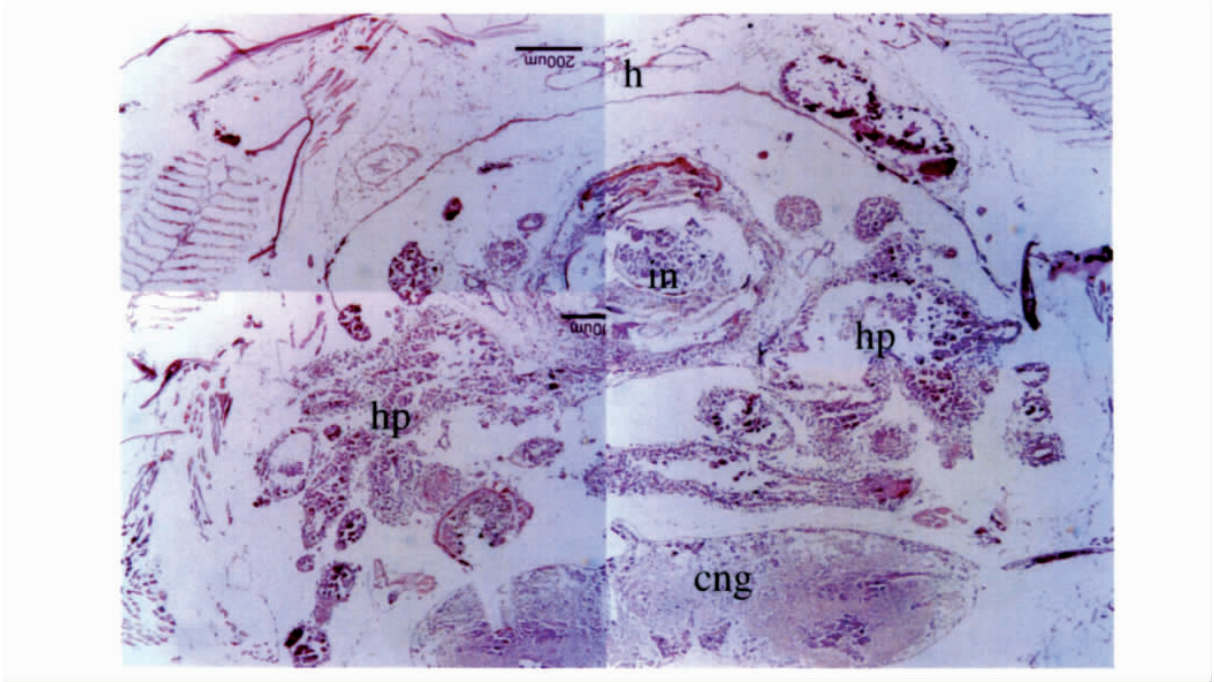

Plate Ⅴ　Transverse section of sex reversed male crab from female at the 5th crab stage

h：heart；hp：hepatopancreas；in：intestine；cng：central nervous ganglion；scale bar：200 μm