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The Characteristics of Sex Differentiation Based on Morphological Traits During the Early Development Stage of the Swimming Crab *Portunus trituberculatus* and Sex Prediction Model Comparison

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Abstract: To investigate the morphological traits of different sexes during the early development stage of the swimming crab *Portunus trituberculatus*, the genetic sex of 405 crablets from stages C I–V was determined using sex-linked molecular markers. The external appearance of each crab was observed, and the morphological traits, including carapace length (CL), full carapace width (FCW), abdomen width (AW), and abdomen length (AL), were measured to compare the differences between the sexes and to develop non-intrusive methods for sex identification. The genetic sex identification demonstrated that the female-to-male sex ratio at each stage did not deviate from 1:1. The gonopores of the females were first observed at stage C I using a scanning electron microscope; however, the shape difference in the abdominal flap between the sexes from stages C I to stage C V were not observed under a dissecting microscope. Correlation analysis showed that AW3 and AW4 were significantly correlated with sex at stage C V. Three methods were used to predict the sex of the crablets according to the morphological measurements; among them, the multinomial logistic regression (MLR) model performed better than the threshold method or the stepwise discriminant analysis, and the accuracy at stage C V increased to 90% when detailed abdominal parameters were included. These results indicate that it is feasible to predict sex during the early development stages of *P. trituberculatus* through morphometric and geometric parameters, which would be helpful for the creation of monosex populations of this species.

Keywords: *Portunus trituberculatus*; sex identification; morphological traits; sex prediction

Key Contribution: (1) The female-to-male ratio of crablets at each stage did not deviate from 1:1. (2) For crablets of *Portunus trituberculatus*, the gonopores of the females were first observed at stage C I using a scanning electron microscope; however, the shape difference in the abdominal flap between the sexes from stage C I to stage C V were not observed under a dissecting microscope. (3) Correlation analysis between morphological traits and sex showed that AW3 and AW4 were significantly correlated with sex at stage C V. (4) The multinomial logistic regression model performs better in sex prediction than the threshold

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method or the stepwise discriminant analysis, and the accuracy at stage C V increased to 90% when detailed abdominal parameters were included.

1. Introduction

The swimming crab *Portunus trituberculatus* is widely distributed in the coastal waters of China, Japan, and Korea [1]. It is one of the most important commercial mariculture species in China, with a production of 1.01×10^5 tons in 2023 [2].

Sex is one of the most valuable traits because sexual dimorphism in growth, size, and other economic characteristics commonly exists in aquaculture animals [3,4]. In crustaceans, morphological sexual dimorphism presents through various aspects such as gonads, gonophores, chelicerae, abdomen, body size, and body weight, which are related to growth and development, environmental adaptation, and immune regulation [5–7]. Sexual differences are evident in *P. trituberculatus*; primarily characterized by faster growth in female crabs and male cannibalism [8]. In addition, female crabs that reach sexual maturity are more profitable with a higher market value due to the accumulation of vitellogenin in the ovary [9].

The development of the sex manipulation technology to create monosex cultures in aquatic animals has been studied extensively in *Oreochromis niloticus* [10], *Oncorhynchus tshawytscha* [11], *Hippoglossus hippoglossus* [12], and *Macrobrachium rosenbergii* [13]. Mating sex-reversed aquatic animals with normal specimens was considered an effective method to produce a monosex population. Studies have shown that the best time for sex manipulation to produce sex-reversed animals in crustaceans is during the early developmental stages. For example, in *M. rosenbergii*, androgenic gland ablation at the early developmental stage of males (at a presumably sexually undifferentiated stage) increased the success rate of sex reversal [14]. A previous study has also shown that to achieve successful masculinization in crabs, the animals used should be no older than the third to the fifth crablet stages [15]. Thus, the development of methods that could discriminate gender at an early developmental stage will increase the efficiency of sex manipulation.

Several methods, including morphology, hormone concentration, molecular techniques, histology, and endoscopic techniques, have been employed to determine the sex of aquaculture animals [16,17]. Among these, morphological sex determination stands out due to its advantages of speed and the mitigation of stress and injury to the aquatic animals [16]. In *P. trituberculatus*, while the sex of mature individuals is easy to distinguish based on their external morphology (such as the shape of the abdomen), there is still no non-intrusive method to detect the sex of crablets at early stages of development.

In this study, we compared the sex-based morphological differences in the carapace and abdomen of the early crablet stages of *P. trituberculatus.* Also, different prediction models were used to develop non-intrusive methods for sex identification during the early development stages, which will be helpful for the implementation of the sex manipulation technology and the creation of monosex cultures of this species.

2. Materials and Methods

2.1. Crab Culture and Sampling

P. trituberculatus larvae from eight wild female crabs hatched simultaneously were collected from cement tanks ($8 \times 4 \times 1.5$ m) at Choupijiang Aquatic Products Limited Company, Zhejiang, China. Subsequently, crablets at stage I (C I) were transferred to the circular plastic tanks with a diameter of 0.65 m and a height of 0.72 m. Plastic mesh pieces were placed in the tank to prevent aggression among crabs and to facilitate the capture of

crablets. The rearing conditions were maintained at a salinity of 20–25 psu and a water temperature of 25–30 °C with continuous aeration. Crabs were fed with brine shrimp, and one third of the water was replaced daily with the removal of uneaten feed. Molting times were recorded, and crabs at each developmental stage were randomly sampled. A total of 81 crablets from each stage were sampled and subjected to morphological observation and measurement using a dissecting microscope (the individual IDs were recorded for subsequent analysis), subsequently, the whole bodies of 61 crabs were stored in absolute ethanol and homogenated to extract DNA for sex identification; while the appendages of the remaining 20 individuals were dissected, preserved in absolute ethanol and homogenated to extract DNA for sex identification, the remaining body parts were used for scanning electron microscope (SEM) observation.

2.2. Morphological Trait Measurement and Observation

External images of the crablets were first captured using a digital camera (HDCE-X5, Yongxin, Ningbo, China) under a dissecting microscope. The morphological indexes (Figure 1), including carapace length (CL), full carapace width (FCW), first abdomen width (AW1), second abdomen width (AW2), third abdomen width (AW3), fourth abdomen width (AW4), fifth abdomen width (AW5), abdomen length (AL), first abdomen length (AL1), second abdomen length (AL2), third abdomen length (AL3), fourth abdomen length (AL4), and fifth abdomen length (AL5), were measured manually using ScopeImage 9.0 software (about 5 min for the measurements of one crab). The abdomen areas of the crablets were divided into triangles or trapezoids as shown in Figure 1B, and artificial variables (S1, S2, S3, S4, S5) were used for area calculation. The gonopores of the crablets at C I–V (crablet stages I–V of) were observed using scanning electron microscopy (SEM).

Figure 1. Schematic diagrams of *P. trituberculatus*: (**A**) Dorsal measurement indices. (**B**) Measurement indices at abdomen. CL (carapace length), FCW (full carapace width), AW1 (first abdomen width), AW2 (second abdomen width), AW3 (third abdomen width), AW4 (fourth abdomen width), AW5 (fifth abdomen width), AL (abdomen length), first abdomen length (AL1), second abdomen length (AL2), third abdomen length (AL3), fourth abdomen length (AL4), fifth abdomen length (AL5), and artificial variables (S1, S2, S3, S4, S5) represent the areas of triangles or trapezoids of different parts of crab abdomen.

2.3. Scanning Electron Microscopy

For SEM analysis, crabs after removal of abdominal flap were first fixed in fresh 3% glutaraldehyde prepared with 0.1 M phosphate-buffered saline (PBS, pH 7.0) for 2 h. The specimens were then washed three times with 0.1 M PBS for 15 min each time. To ensure complete dehydration, the specimens were passed through a graded ethanol series (30%, 50%, 70%, 80%, 90%), with each step lasting for 15 min, followed by soaking twice in anhydrous ethanol for 20 min each time. Then, the specimens were soaked in a series of mixed solution (ethanol mixed with tert-butanol in ratios of 3:1, 1:1, 1:3, respectively), each bath lasting for 15 min, followed by soaking in pure tert-butanol for 20 min. Subsequently, the specimens were immersed in an appropriate amount of pure tert-butanol, frozen at

−20 °C for more than 1 h, and vacuum-dried for more than 24 h. Then, they were sputter coated with gold using a Hitachi E-1010 sputter coater (Hitachi Ltd., Tokyo, Japan), and observed under an S-3400 scanning electron microscopy (Hitachi Ltd., Tokyo, Japan) at 10 kV.

2.4. Identification of Genetic Sex

The genetic sex of each crablet was determined using the Kompetitive Allele Specific PCR (KASP) method previously developed by our laboratory [18]. In brief, genomic DNA was extracted using EZNA® Tissue DNA Kit (Omega, GA, USA) according to the manufacturer's instructions, and the KASP reactions were conducted on a LightCycler480 instrument (Roche, Basel, Switzerland) using primers PtS7FAM, PtS7HEX, and PtS7C (Loci: Ptr67655). PCR of 10 µL reaction volume without genomic DNA was set as control.

2.5. Data Analysis

Measurements of the morphological indices are expressed as mean values with the standard errors. All data were analyzed using Microsoft Excel 2013 and IBM SPSS 22.0. Prior to statistical analysis, normality and homoscedasticity tests were assessed using the Levene test. Independent *t*-tests were performed to identify significant difference between sexes. The correlation analysis between morphological traits and sex was conducted using Spearman's rank correlation coefficient. Additionally, the chi-square (χ^2) test was used to determine whether the ratio of males to females deviated from an equilibrium of 1:1 at each developmental stage.

Three methods were used to construct a sex prediction model based on the morphological measurements. (1) Threshold method: the values of abdomen AW were normalized first to find a formula that best distinguishes females from males. The threshold calculated by this formula for each stage was then used to assign the sex of the crablets. (2) Stepwise discriminant analysis method: to construct the discriminant function equation, the values of different abdomen AW were normalized by AL and then subjected to stepwise discriminant analysis using SPSS 20.0. (3) Multinomial logistic regression (MLR) analysis: this analysis was conducted on the morphological measurements and indices (e.g., FWC, AL, AW1/AW5, S1) using MATLAB 9.10. When the response variable was categorical, the model was specified as follows:

$$\ln \frac{P}{1-P} = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \cdots + \beta_k x_k, (0 < P < 1) \quad (1)$$

where P, x, and β are the predicted probability, explanatory variable, and regression coefficient optimized by a maximum likelihood estimation, respectively.

The results of sex assignment using the three methods above were compared to the genetic identification to evaluate their accuracy.

3. Results

3.1. Identification of Genetic Sex at Different Developmental Stages

The genetic sex of 405 crablets (81 crablets at each stage) was successfully identified (Figure 2). In the scatter fluorescence plot based on genotypic data; female individuals clustered near the *Y*-axis, while male individuals clustered near the diagonal. No amplification was observed in the control group.

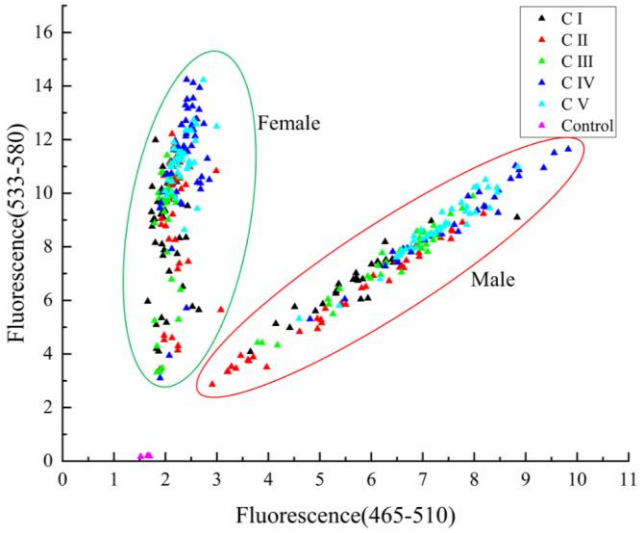


Figure 2. Genetic sex identification of crablets at different development stages.

Although the number of males and females varied at different stages, the female-to-male sex ratio at each stage did not deviate from 1:1 (Table 1).

Table 1. Analysis of genetic sex at different developmental stages.

Stage	Number	Female	Male	Ratio (Female/Male)	χ^2
C I	81	36	45	0.80	1.000
C II	81	40	41	0.98	0.012
C III	81	42	39	1.08	0.111
C IV	81	49	32	1.53	3.568
C V	81	39	42	0.93	0.111
Total	405	206	199	1.04	0.121

Note: The significance level is set at 0.05, with a corresponding critical value of 3.841.

3.2. Analysis of Morphological Traits in Crablets at Different Developmental Stages

The molt interval during the early stage of crablets was 3–4 days, and the molting and sampling times for crablets at the five stages are outlined in Table 2. A comparative analysis of morphological indices was conducted based on the results of genetic sex identification. The morphological traits of females and males increased gradually with crablet molting (Table 3). From stage C I to C V, no significant differences were observed in any of the morphological measurements. However, at stage C V, AW3 and AW4 showed significant differences between sexes ($p < 0.01$). Data analysis after normalization also revealed that AW3/AL and AW4/AL exhibited significant differences between sexes at stage C V ($p < 0.01$), whereas other morphological traits did not show significant differences between sexes (Table 4).

Table 2. The molting and sampling times at different stages of *P. trituberculatus* crablets.

Stage	C I	C II	C III	C IV	C V
Molting time		PJ3	PJ6	PJ10	PJ14
Sampling time	PJ2	PJ4	PJ7	PJ11	PJ15

C I–V, crablet stage I–V; PJ, post juvenile crab.

Table 3. Analysis of morphological traits of crablets at different developmental stages.

Traits	Sex	C I	C II	C III	C IV	C V
FCW (mm)	Female	5.11 ± 0.24	7.20 ± 0.32	9.94 ± 0.59	16.79 ± 1.05	22.00 ± 1.18
	Male	5.12 ± 0.28	7.07 ± 0.30	9.96 ± 0.51	17.10 ± 0.92	21.50 ± 1.22
CL (mm)	Female	3.25 ± 0.13	3.88 ± 0.23	5.03 ± 0.23	8.20 ± 0.50	10.67 ± 0.60
	Male	3.24 ± 0.20	3.83 ± 0.23	4.96 ± 0.29	8.36 ± 0.47	10.46 ± 0.56
AW1 (mm)	Female	0.39 ± 0.03	0.41 ± 0.04	0.52 ± 0.06	0.85 ± 0.09	1.03 ± 0.09
	Male	0.39 ± 0.02	0.40 ± 0.39	0.50 ± 0.05	0.87 ± 0.09	1.02 ± 0.08
AW2 (mm)	Female	0.50 ± 0.03	0.55 ± 0.05	0.70 ± 0.07	1.17 ± 0.11	1.48 ± 0.13
	Male	0.49 ± 0.02	0.53 ± 0.05	0.70 ± 0.07	1.20 ± 0.10	1.45 ± 0.12
AW3 (mm)	Female	0.63 ± 0.04	0.67 ± 0.05	0.87 ± 0.08	1.49 ± 0.14	1.96 ± 0.16 **
	Male	0.66 ± 0.04	0.65 ± 0.01	0.87 ± 0.07	1.49 ± 0.17	1.81 ± 0.15 **
AW4 (mm)	Female	0.83 ± 0.03	0.89 ± 0.05	1.20 ± 0.10	2.10 ± 0.21	2.78 ± 0.22 **
	Male	0.85 ± 0.05	0.89 ± 0.06	1.16 ± 0.08	2.04 ± 0.22	2.46 ± 0.23 **
AW5 (mm)	Female	1.15 ± 0.01	1.72 ± 0.08	2.56 ± 0.21	4.58 ± 0.38	6.04 ± 0.46
	Male	1.17 ± 0.01	1.69 ± 0.10	2.53 ± 0.19	4.65 ± 0.34	5.91 ± 0.42
AL (mm)	Female	1.45 ± 0.08	1.73 ± 0.13	2.38 ± 0.22	4.18 ± 0.46	5.55 ± 0.45
	Male	1.46 ± 0.07	1.69 ± 0.13	2.32 ± 0.18	4.20 ± 0.35	5.49 ± 0.40

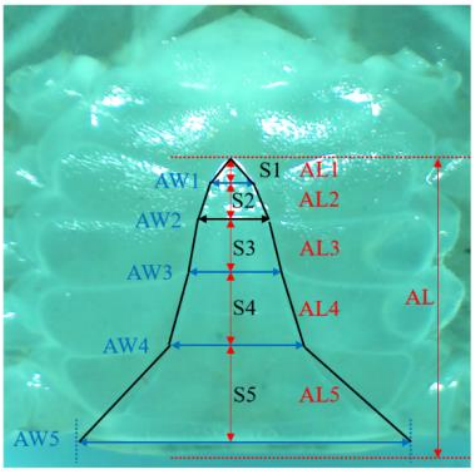
Note: "**" indicated significant differences between male and female ($p < 0.01$).

Table 4. Analysis of standardized morphological traits of crablets at different developmental stages.

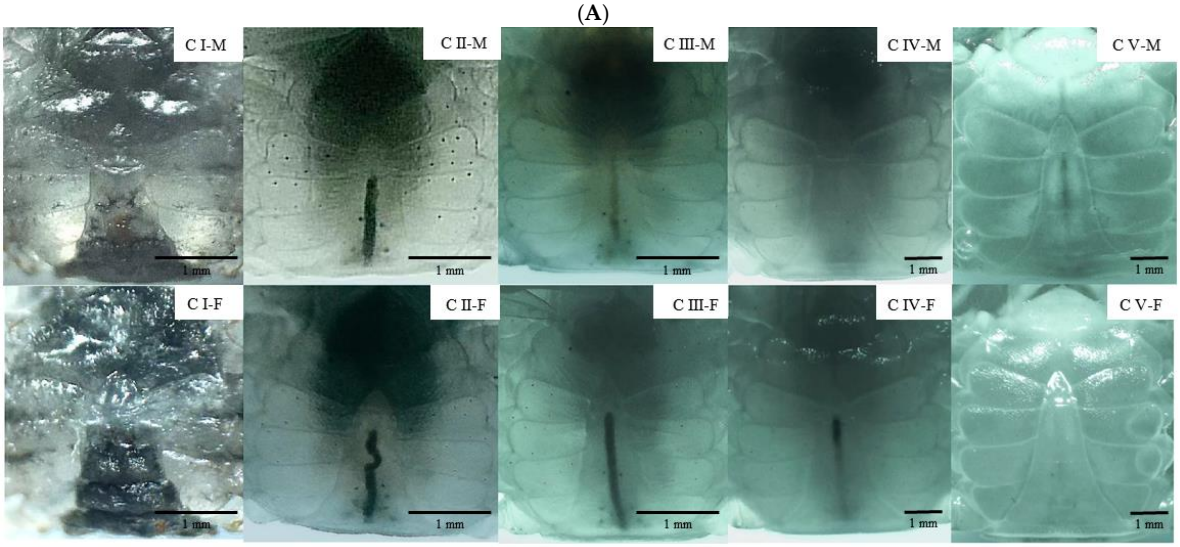
Traits	Sex	C I	C II	C III	C IV	C V
CL/FCW	Female	0.64 ± 0.02	0.54 ± 0.02	0.51 ± 0.02	0.49 ± 0.02	0.49 ± 0.01
	Male	0.63 ± 0.04	0.54 ± 0.03	0.50 ± 0.02	0.49 ± 0.01	0.49 ± 0.01
AW1/AL	Female	0.27 ± 0.02	0.24 ± 0.02	0.22 ± 0.02	0.21 ± 0.02	0.19 ± 0.01
	Male	0.27 ± 0.02	0.24 ± 0.02	0.22 ± 0.02	0.21 ± 0.02	0.19 ± 0.01
AW2/AL	Female	0.35 ± 0.02	0.32 ± 0.03	0.30 ± 0.03	0.28 ± 0.03	0.27 ± 0.02
	Male	0.34 ± 0.02	0.32 ± 0.03	0.30 ± 0.03	0.29 ± 0.03	0.27 ± 0.02
AW3/AL	Female	0.44 ± 0.03	0.39 ± 0.04	0.37 ± 0.03	0.36 ± 0.04	0.35 ± 0.02 **
	Male	0.45 ± 0.04	0.39 ± 0.03	0.38 ± 0.03	0.36 ± 0.05	0.33 ± 0.03 **
AW4/AL	Female	0.58 ± 0.04	0.52 ± 0.04	0.51 ± 0.04	0.51 ± 0.05	0.50 ± 0.03 **
	Male	0.58 ± 0.04	0.53 ± 0.04	0.50 ± 0.04	0.49 ± 0.05	0.45 ± 0.04 **
AW5/AL	Female	0.80 ± 0.04	1.00 ± 0.06	1.08 ± 0.07	1.10 ± 0.07	1.09 ± 0.05
	Male	0.80 ± 0.04	1.00 ± 0.07	1.09 ± 0.06	1.11 ± 0.07	1.08 ± 0.07

Note: "**" indicates significant differences between male and female ($p < 0.01$).

Based on the results of genetic sex identification, the abdominal morphology of females and males was compared. Results showed that the abdominal morphology of female and male crablets from C I–V were not much different under the dissecting microscope observation (Figure 3). However, using scanning electron microscopy (SEM), a pair of symmetrical gonopores was observed near the edge of the abdomen at the third thoracic segment in females across all stages (C I–V), whereas no gonopores were found at the same position in males (Figure 4).



(**A**)



(**B**)

Figure 3. Abdominal morphology of *P. trituberculatus* crablets. (**A**) Example of abdomen measurement parameters (CV-F). (**B**) The external morphology of the abdomen at different development stages of male and female *P. trituberculatus* crablets. F, female; M, male. Note: measurement indices of abdomen in diagram (**A**) were the same as Figure 1B.

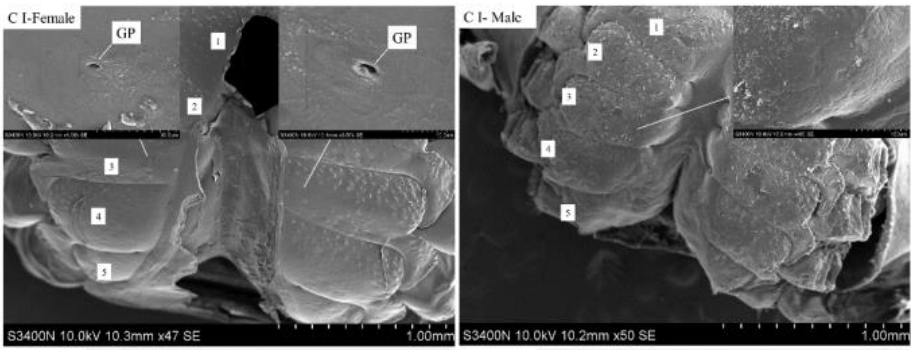


Figure 4. *Cont.*

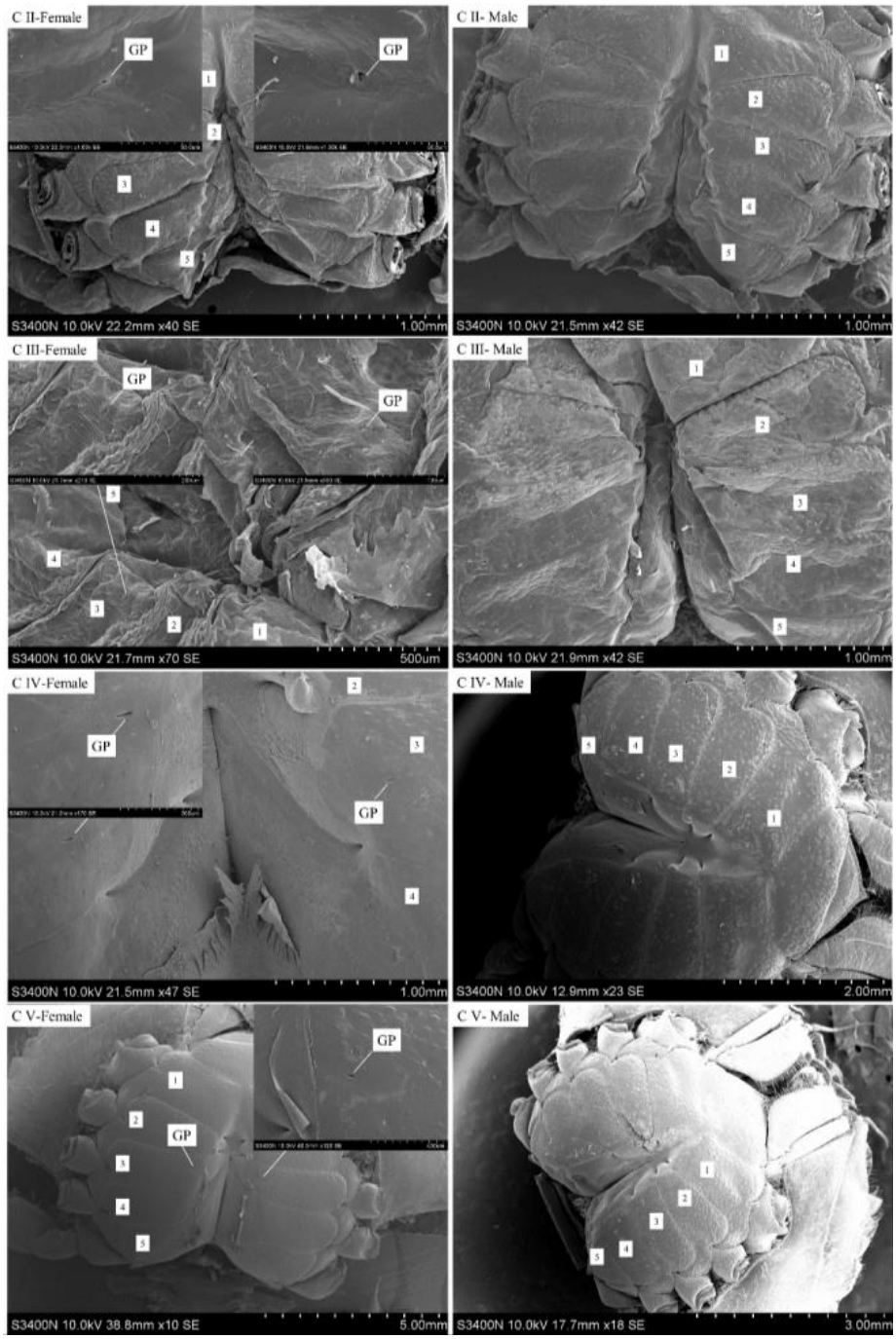


Figure 4. The gonopores characteristics of juvenile female crabs in C I–C V. GP: gonopores; 1–5: the first to fifth thoracic sternite.

3.3. Correlation Analysis Between Morphological Traits and Sex

Correlation analysis of the eight morphological traits with sex revealed that AW3 was significantly correlated with sex at stage C II ($p < 0.05$), while both AW3 and AW4 were significantly correlated ($p < 0.01$) with sex at stage C V (Table 5). These findings suggest that AW3 and AW4 can be used as the primary candidate morphological indicators for sex determination.

Table 5. Correlation coefficients between the morphological traits and sex of crablets at different developmental stages.

Stage	FCW	CL	AW1	AW2	AW3	AW4	AW5	AL
C I	−0.003	−0.039	−0.024	−0.176	0.286	0.140	0.175	−0.027
C II	−0.210	−0.127	−0.076	−0.152	−0.241 *	−0.061	−0.149	−0.146
C III	0.049	−0.085	−0.148	0.012	0.001	−0.193	−0.020	−0.100
C IV	0.152	0.126	0.101	0.125	−0.012	−0.161	0.080	0.055
C V	−0.199	−0.194	−0.055	−0.140	−0.437 **	−0.589 **	−0.146	−0.057

Note: "*" indicates significant differences ($p < 0.05$), "**" indicates significant differences ($p < 0.01$).

3.4. Construction and Test of Sex Prediction Models

Given that the most significant differences in morphological traits between sexes were observed at stage C V, the values of morphological traits at this stage were preferentially used to construct sex prediction models.

(1) Threshold method. A comparison of morphological indices and correlation analysis between morphological traits and sex revealed that AW3 and AW4 were significantly correlated with sex. Therefore, AW3 and AW4 at stage C V were normalized by AL and FCW to construct the threshold model. First, we tested whether the normalized data followed a normal distribution. Then, we plotted the normal distribution curves for male and female crabs and used the x-coordinate of their intersection as the threshold to assign individuals to different sexes. The reliability of the threshold method was assessed using 405 individuals from five development stages. When compared with genetic sex identification, the AW4/FCW index performed the best for sex assignment, and the overall accuracy rate of sex assignment increased from C I to C V based on thresholds calculated using AW4/FCW (Table 6), with an accuracy rate of 87.18% for female and 73.81% for males at stage C V.

Table 6. The accuracy of crablet sex identification using threshold method at different developmental stages.

Traits of Different Stage	Genetic Sex	Threshold	Identification Accuracy (%)
$C_1\left(\frac{AW4}{FCW}\right)$	Female Male	0.1700	26.09 63.64
$C_2\left(\frac{AW4}{FCW}\right)$	Female Male	0.1278	25.00 68.42
$C_3\left(\frac{AW4}{FCW}\right)$	Female Male	0.1199	60.00 92.31
$C_4\left(\frac{AW4}{FCW}\right)$	Female Male	0.1186	75.51 48.39
$C_5\left(\frac{AW4}{FCW}\right)$	Female Male	0.1200	87.18 73.81

(2) Stepwise discriminant analysis method: One morphological trait and two normalized Variables (AL, AW2/AL and AW4/AL) of stage C V were identified as contributors to Fisher's linear discriminant function for sex identification based on stepwise discriminant analysis. The discriminant equations were established as follows:

$$F1 = 55.520 \times AL + 727.379 \times AW2/AL + 422.610 \times AW4/AL - 358.368$$

$$F2 = 53.907 \times AL + 766.894 \times AW2/AL + 363.707 \times AW4/AL - 331.964$$

If F1 > F2, the candidate was categorized as female; otherwise, it was male. The reliability assessment of the discriminant equations was conducted using 405 individuals from five developmental stages (Table 7). When compared with genetic sex identification, the overall accuracy of sex assignment increased from stages C I to C V, with an accuracy rate of 84.62% for females and 80.95% for males at stage C V.

Table 7. The accuracy of crablet sex identification using stepwise discriminant analysis at different developmental stages.

Stage	Genetic Sex	Number	Predicted Sex		Identification Accuracy (%)	Total Discriminant Accuracy (%)
			Female	Male		
C I	Female	36	1	35	2.78	54.32
	Male	45	2	43	95.56	
C II	Female	40	0	40	0.00	50.62
	Male	41	0	41	100.00	
C III	Female	42	0	42	0.00	48.15
	Male	39	0	39	100.00	
C IV	Female	49	17	32	34.69	53.09
	Male	32	6	26	81.25	
C V	Female	39	33	6	84.62	82.72
	Male	42	8	34	80.95	

(3) Multinomial logistic regression (MLR) analysis. Multinomial logistic regression model for different developmental stages were analyzed under both known and unknown AL1-5 conditions (models were provided in the Supplementary Material). The MLR analyses were conducted for 29 combinations of elements with known AL1-5 and 19 combinations without AL1-5. When compared with genetic sex identification, the accuracy of sex assignment was higher than that of the threshold method or the stepwise discriminant analysis, reaching 86.96% for female and 81.82% for male at stage C I without AL1-5. When AL1-5 data were added, the accuracy of sex assignment increased to 94.87% for female and 92.86% for male at stage C V (Table 8).

Table 8. The accuracy of crablet sex identification using multinomial logistic regression at different developmental stages.

		Identification Accuracy (%)	
Stage	Genetic Sex	Unknown for AL1-5 Segments (Unable to Obtain Values for S1–S5)	Known for AL1-5 (S1–S5 Values Can Be Obtained)
C I	Female	86.96%	/
	Male	81.82%	
C II	Female	72.22%	/
	Male	76.32%	
C III	Female	67.50%	80.00%
	Male	63.89%	72.22%
C IV	Female	85.71%	87.76%
	Male	61.29%	61.29%
C V	Female	89.74%	94.87%
	Male	85.71%	92.86%

4. Discussion

Crustaceans are important economic species in aquaculture, often exhibiting sexual dimorphisms that result in differences in morphological, physiological, and behavioral characteristics and economic value [19]. For example, the monosex culture of all-male populations of *M. rosenbergii* is more profitable than mixed or all-female cultures due to faster growth [13], and sexual manipulation of this species has proven effective in generating monosex populations for aquaculture through the androgenic gland (AG), a crustacean-specific endocrine gland [20].

The monosex culture of female *P. trituberculatus* holds promising commercial potential in the market. Currently, research on sexual manipulation technologies to produce all-female populations of *P. trituberculatus* is still in progress. Farmers typically use the "sex grading" method to remove males from the pond, this process is usually conducted 3–4 month after pond culture, when there is a noticeable difference in the abdominal morphology between males and females (the female's abdominal flap is nearly round, while the male's is triangular). However, this method is laborious and inaccurate (farmers need to recapture the crabs from the earth pond, distinguish the sexes visually, and then put the female back into the pond and remove the male, and the randomness of recapture also cannot guarantee that only females remain in the pond). Studies on the morphological characteristics of *P. trituberculatus* during the early development stages could facilitate the development of a non-intrusive sex identification method at early stage (for example, before the crablets are transferred from hatchery falilities to earth ponds) based on morphological traits, which will reduce the cost of pond culture and improve the success rate of sex manipulation to produce monosex populations.

The position of gonopores on the third (female) or fifth (male) pereopods serves as a reliable indicator of sex in all malacostracan crustaceans [21]. In *Scylla paramamosain*, the presence of gonopores were observed under SEM at stage C I [6]. Using SEM, we also identified a pair of symmetrical gonopores near the edge of the abdomen covered by abdominal flap in all stages (C I–V) of the *P. trituberculatus* females, indicating that the sex differentiation occurs before the crablet stage I. Lee et al. [22] reported the presence of female gonoducts in the megalopa stage of *Eriocheir japonicus*, implying that sex differentiation may begin as early as the zoeal stage in decapods. However, as a typical sex indicator, it is difficult to visually detect gonopores at an early crablet stage. In *S. paramamosain*, the presence of a gonopore and forked pleopod could be detected in the carapace width range 5.01–7.50 mm using an optical microscope, although their visibility was unclear. The gonopods were more clearly visible in individuals with a carapace width >10 mm (±C V stage) [23]. Furthermore, separating the abdominal flap to detect gonopores will also cause damage to the crablet. Therefore, while gonopores can serve as an indicator for early sex discrimination, the method will cause damage to the crablet and is not suitable for non-destructive sex determination in crablets.

In *S. paramamosain*, the female's abdominal flap was wider than the male's at stage C V when observed using an optical microscope with 8–20×magnifications [6,23]. In this study, no difference in the abdominal flap was observed between sexs in *P. trituberculatus* at stage C V under a dissecting microscope (Figure 3). However, measurements of morphological traits revealed that AW3 and AW4 began to exhibit significant sexual dimorphism at stage C V ($p < 0.01$). The sexual dimorphism in abdominal shape occurred earlier in *P. trituberculatus* than in *S. paramamosain*, where significant differences were first observed at stage C VI [6].

Morphometric and geometric indices have been used to predict body weight, meat yield, and sex in aquatic animals in order to build non-intrusive and rapid detection methods, improving the efficiency of fisheries management [24–28]. In this study, we employed three methods to predict the sex of crablets based on morphological measurements and

compared the accuracy of these methods. Among these, the threshold method and the stepwise discriminant analysis, which rely on a few important gender-related parameters (eg: AW3, AL), demonstrated low accuracy for sex identification of crablets at stages C I–IV, and the accuracy improved to 80% at stage C V, which was still lower than the MLR model constructed using comprehensive morphological parameters. The accuracy of the MLR model at stage C V exceeded 85%, especially when abdominal parameters of AL1-5 and related geometric indices (S1–S5) were included, the accuracy at stage C V exceeded 90%, indicating that abdominal indices play important roles in sex identification. In addition, the performance of the MLR model at stages C I–C IV was superior to that of the other two methods, with accuracy rates of sex assignment ranging from 61% to 86%. These results suggest that it is feasible to predict the sex of *P. trituberculatus* during the early development stages using morphometric and geometric parameters, with the MLR model outperforming the threshold method and stepwise discriminant analysis. However, the samples used in this study were crablets collected from a hatchery with precise developmental stages, and the applicability of the discrimination method established in this study on the sex discriminate of *P. trituberculatus* crablets collected at different sampling times or from different environments requires further investigation.

In addition, although the accuracy for sex prediction at stage C V increased to 90% with the MLR model, the accuracy rate for sex prediction at stages C I–C IV were still low, and it take times to measure the phenotype of crablets. Improving the accuracy and efficiency of phenotype measurement, as well as developing a more precise prediction model may help increase the accuracy of sex prediction at stages C I–C IV. In recent years, advancements in automation and artificial intelligence, computer vision, and machine learning have been extensively applied in aquaculture, significantly enhancing the accuracy and efficiency of data collection and analysis in fisheries research [29,30]. The promising results of our study, combined with these technologies could help develop more accurate and automated methods for predicting the sex for crablets at an early development stage of *P.trituberculatus* based on morphometric and geometric characters of the abdomen and facilitate the creation of monosex populations of this species.

5. Conclusions

In this study, we analyzed 405 *P. trituberculatus* crablets (81 crablets from stage C I to C V). Genetic sex identification using KASP revealed that the female-to-male sex ratio at each stage did not deviate from 1:1. The gonopores of females were first observed at stage C I using a scanning electron microscope. Correlation analysis between morphological traits and sex showed that AW3 and AW4 were significantly correlated with sex at stage C V. Three methods were used to predict sex of crablets based on the morphological measurements. Among these, the threshold method and the stepwise discriminant analysis that rely on a few important gender-related parameters (e.g.,: AW3, AL) have a low accuracy for sex identification, while the MLR model constructed using comprehensive morphological parameters performed better, with accuracy at stage C V increased to 90% when detailed abdominal parameters were included.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fishes10010008/s1, Table S1. Variable and corresponding element used with known AL1-5; Table S2. Variable and corresponding element used with unknown AL1-5.

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Institutional Review Board Statement: This study was approved by the Ethics Committee of Ningbo University (no. 20190410-042) and conducted according to relevant national and international guidelines.

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