



ECOSYSTEMS

Native and non-native species of *Litopenaeus* Pérez-Farfante, 1969 (Crustacea: Penaeidae) from the East Atlantic: Geometric morphometrics as a tool for taxonomic discrimination

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Abstract: The shape of the secondary sexual characters is, traditionally, used to discriminate the marine shrimps at the species level. However, the qualitative evaluation of structures that are morphologically variable in the taxonomy of species can favor misunderstandings and misidentifications. These possibilities of taxonomic inaccuracies are especially alarming when there is a need to evaluate the invasion of introduced species. The present study used geometric morphometric analyses to identify differences in the cephalothorax shape that would help discriminate the native and non-native species of *Litopenaeus* of the South American coast. The comparative morphology analysis was conducted using the right profile of adult males' cephalothorax of *L. schmitti*, captured in the natural environment, and *L. vannamei* captured in the natural environment or grown in shrimp farms. In intraspecific evaluation, it was not possible to distinguish the specimens of *L. vannamei* that were grown in shrimp farms from those acclimated to the natural environment. However, significant interspecific differences in shape were found in the shape of this body structure. Additionally, the base position of the first rostral spine to the tip of the hepatic spine is indicated as a characteristic that can be used to distinguish these two species by eye in the field.

Key words: white shrimp, morphological comparison, exotic species, bioinvasion, taxonomy.

INTRODUCTION

The species identification of many Penaeidae shrimps, within the same genus, is primarily characterized by the shape of the secondary sexual characters, due to their great anatomical diversity between the species and the tremendous morphological conservation of the other characters (Dall et al. 1990, Lavery et al. 2004, Pérez-Farfante & Kensley 1997). Although these structures are easy to visualize and to distinguish interspecifically, they may be variable within species, since their shape changes along the ontogenetic development (Pérez-Farfante

1970). The use of only one morphological structure, of variable tendency, in the species identification can promote confusion and incorrect identifications. Taxonomic imprecision is especially alarming when there is a need to evaluate introduced species' invasion, but it is difficult to distinguish them from their native counterparts.

In several regions of the world, shrimp *Litopenaeus vannamei* (Boone, 1931) is one of the most widely used species in the production of cultured marine shrimps (Freitas et al. 2007), due to its characteristics of high adaptation,

rapid growth, and high survival rates to adverse environmental conditions (Briggs et al. 2004). Originally from the Eastern Pacific, this species is used by shrimp farmers throughout the Western Atlantic (Tavares 2011). Its extensive cultivation facilitated the possibility of escape to the natural environment, allowing individuals to be found co-occurring with their native congener, *L. schmitti* (Burkenroad, 1936) (Barbieri & Melo 2006, Loebmann et al. 2010, Santos & Coelho 2002). Although there are numerous escape cases, the population status of *L. vannamei* in the natural environment in the Atlantic coastline of South America is still uncertain.

In the search for alternatives that increase the accuracy of identifications and aid in discriminating the native and non-native species, new and more resolutive methods are needed to quantitatively describe the body shape of animals, among which geometric morphometric techniques stand out (Rohlf & Marcus 1993). These techniques have their roots in traditional morphometrics, allowing statistical inference, however, preserving information about the geometry (Adams et al. 2004) of the desired body structure. Besides, it allows the quantification of characteristics that are difficult to measure with traditional techniques.

The presence of rigid body structures in marine shrimps makes it a useful organism for the use of geometric morphometric techniques, especially those based in anatomical landmarks. Since it fulfills the prerequisites and is involved in multiple functions in the shrimps, the cephalothorax is considered a structure not only well recognized for taxonomic distinction but also adequate to investigate morphological responses to contrasting environments (Accioly et al. 2013, Bissaro et al. 2013, Sganga et al. 2016, Zimmerman et al. 2011). Species can show different alterations in the basic structure of the cephalothorax, reflecting signs of sexual

dimorphism (Accioly et al. 2013, Alencar et al. 2014, Sganga et al. 2016, Moraes et al. 2020), environmental adaptation (Bissaro et al. 2013, Zimmerman et al. 2011), or as a reflection of genetic differences resulting from selective pressures (Helms et al. 2015).

Species widely cultivated, and present invasion records are of particular interest for monitoring. Such as *L. vannamei* invasion in South America. Techniques and procedures that allow the identification of specimens captured in the natural environment and distinguishment whether these are coming from accidental releases, or if they are completing their life cycle in the natural environment are thus emergent. Therefore, this study aimed to discriminate between the non-native species *Litopenaeus vannamei* and the native congener from western Atlantic *L. schmitti*, with the use of landmark-based geometric morphometrics. Thus, contributing with information that could help correctly assess the population status of these species.

MATERIALS AND METHODS

Specimens of *Litopenaeus schmitti* and *L. vannamei* were collected from the natural environment, in the coastal strip of the Baía Formosa municipality (06°21'49,302''S 35°00'36,830''W), Northeastern Brazil. Samplings were performed monthly, between March of 2013 and February of 2015, using a motorized fishing boat equipped with a single-rig trawl. Additionally, *L. vannamei* specimens from shrimp farms were obtained by purchasing an equal number of individuals cultivated by three independent producers in the State of Rio Grande do Norte, Northeast of Brazil. Immediately after capture, all animals were cryo anesthetized on ice and subsequently fixed in 95% ethanol.

The identification of the specimens was based on Pérez-Farfante (1969, 1988), and the sex was determined from the observation of the presence (males) or absence (females) of the adult male sexual character (petasma), located in the first pair of pleopods. In this study, only specimens with intact cephalothorax and rostrum were used. In order to avoid the effects of body shape variation of ontogenetic allometry and sexual dimorphism, only male shrimps from the same adult cohort (animals that were sexually mature morphologically) were used. Morphological sexual maturity was defined by the presence of united and fully developed endopodites (Pérez-Farfante 1969). Thirty specimens of *Litopenaeus schmitti*, thirteen of *L. vannamei* obtained in the natural environment, and thirty of *L. vannamei* obtained in shrimp farms were used for morphometric investigation, considering the species or origin of the animals as distinct groups.

All specimens had the right cephalothorax profile (unpaired symmetry; Cardini 2016) photographed by the same researcher (Moraes, A.B.), using a Nikon Coolpix L810 (16.1 megapixels)

digital camera, at maximum resolution, coupled to a tripod. The distance from the lens to the body structure, the zoom constancy, and the central position in the picture frame were standardized. Tests of error measurement of photo capture and positioning of landmarks (Viscosi & Cardini 2011) were performed before the investigations for each species and a group of origin (*results not shown*).

The Software tpsDig 2.26 (Rohlf 2006) was used for digitizing eight anatomical landmarks selected based on morphometric homology criteria according to the Bookstein's classification (1982). Additionally, 11 anatomical semi-landmarks were digitized in the cephalothorax (Fig. 1, Table I). The semi-landmarks were used to represent homologous curves to establish a geometric homology between corresponding semi-landmarks through the samples (Gunz & Mitteroecker 2013). The coordinates of the semi-landmarks were aligned using the curves tool of Tpsdig 2.26 software (Rohlf 2006) to draw a curve over the edge of the desired structure. Subsequently, a curve resampling was performed to distribute

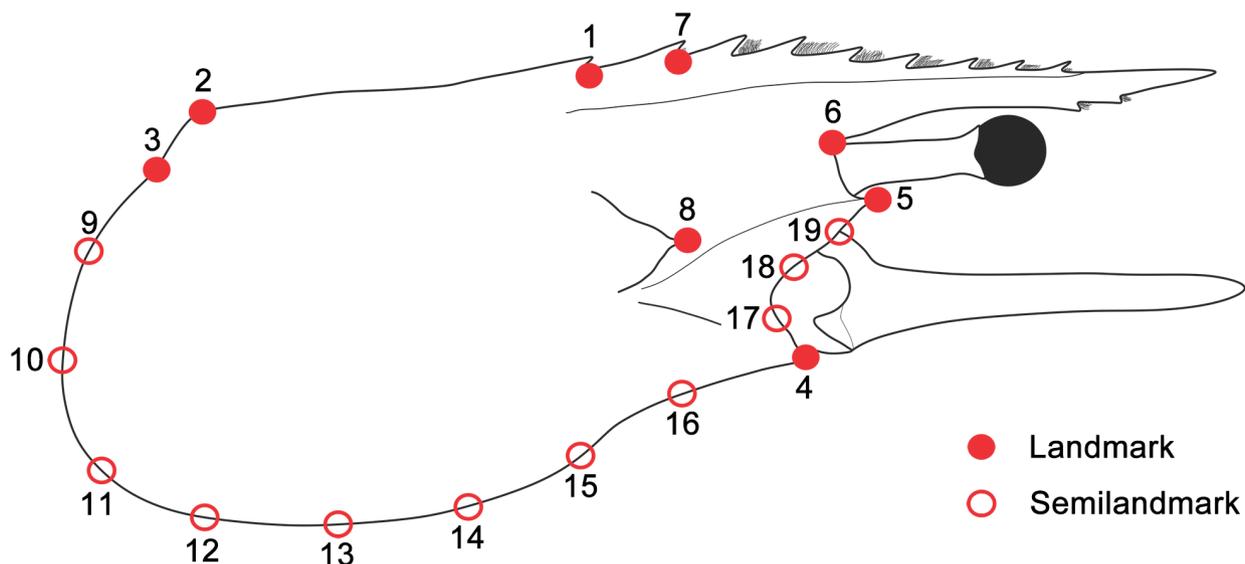


Figure 1. Location of landmarks and semi-landmarks on the cephalothorax right profile for investigation of geometric morphometry. Illustration from *Litopenaeus vannamei* (Boone, 1931).

equal numbers of semi-landmarks on the curve using the equidistance criterion. It used eight semi-landmarks for the cephalothorax's posteroventral edge, between the landmarks 3 and 4, and three semi-landmarks for the anterior border between landmarks 4 and 5. This procedure aims to eliminate arbitrary spacing effects of digitized semi-landmarks in curves.

The coordinates of the landmarks were subjected to a Generalized Superimposition of Procrustes Analysis (GPA) (Rohlf & Slice 1990) in the software MorphoJ 1.06 D[®] (Klingenberg 2011). This technique corrects the effects of scaling, rotation, and positioning of the specimens, maintaining only the shape settings (Adams et al. 2013, Dryden & Mardia 1998).

Table I. List of landmark descriptions used in the investigation of the variation of the cephalothorax shape of *Litopenaeus schmitti* (Burkenroad, 1936) and *L. vannamei* (Boone, 1931).

Landmark	DESCRIPTION
1	Anterior base of the epigastric tooth
2	Distal point of the posterior dorsal margin of cephalothorax
3	Distal point of the posterodorsal margin of the cephalothorax
4	Proximal point of the antero-ventral margin of the cephalothorax
5	Antennal spine tip
6	Apex of the orbital cavity
7	Anterior base of the first tooth of the rostral spine
8	Hepatic spine tip
9-16	Marginal semi-landmarks of the posteroventral angle
17-19	Semi-landmarks of the margin between the antennal and Pterygostomian spines

A multivariate regression (grouped between species and origin) of the coordinates of Procrustes (shape) on centroid size (size) were used to analyze the variation of shape by the allometric effect (Drake & Klingenberg 2008). Static or ontogenetic allometric effects (Cock 1966) are not desirable in this study. In order to avoid them, The allometric correction procedure proposed by Alencar et al. (2014) was used to compare the body shape of each species and origin group. For this, the residuals of the multivariate regression were used for the subsequent statistical analyses and evaluations of variation of the shape.

A Canonical Variates Analysis (CVA) was performed using the grouping factors 'species' vs. 'origin of specimens' to investigate the degrees of similarity between body shapes within the morpho-space of all data obtained. Then, a Discriminant Function Analysis (DFA) was applied to evaluate which variations in shape could reliably distinguish the species and, separately, the origin of the specimens. Probability values were computed from the permutation test ($n = 10,000$) based on Procrustes distances in the comparisons performed in CVA and DFA. The values of Procrustes distance were preferred over Mahalanobis distance because the first is the measure of the absolute amount of variation of the shape, while the second is a relative measure of the variation of the shape (Klingenberg & Monteiro 2005). Finally, we analyze cross-validation matrix percentages obtained in the DFA comparisons.

Procedures of Generalized Superimposition of Procrustes, allometric correction, and multivariate analyses were performed in software MorphoJ 1.06 D[®] (Klingenberg 2011). Transformation grids and comparative wireframe graphs (Klingenberg 2013) were generated from the scores obtained in the DFA and the canonical axes of the CVA.

RESULTS

The CVA provided a visual display of the morphospace considering the two groups of *L. vannamei* and *L. schmitti*, with component 1 representing 92.31% of the total variance (Fig. 2). The majority variation of shape is interspecific, although by the second axis, even if in lesser proportion, it is possible to identify that *L. vannamei* possesses a particular morphological variation distinct between cultured and natural specimens.

The results of the CVA are reaffirmed by the DFA, which confirms both the statistical difference of shape between *L. schmitti* and *L. vannamei*, and the statistical similarity in shape between the groups of *L. vannamei* from the natural environment and cultivated in shrimp farms. Among the species, discriminant function

analysis determined statistical difference between *L. schmitti* and *L. vannamei* (procrustes distance = 0.0293; $P < 0.01$) (Table II, Fig. 3a). For this relationship, we obtained 96.42% of correct signaling of the cross-validation matrix for *L. schmitti* and 100% for *L. vannamei*. In the evaluation regarding the groups of origin, the DFA showed statistical difference in the two interspecific relationships, revealing greater similarity when both species are of the natural environment (distance of procrustes = 0.0273; $P = 0.037$) (Table II, Fig. 3b), and lower similarity when the non-native species is from shrimp farms (distance from procrustes = 0.0308; $P < 0.01$) (Table II, Fig. 3c). The cross-validation matrix indicated higher percentages of correct classification when cultured *L. vannamei* individuals were compared, 71.42% for *L.*

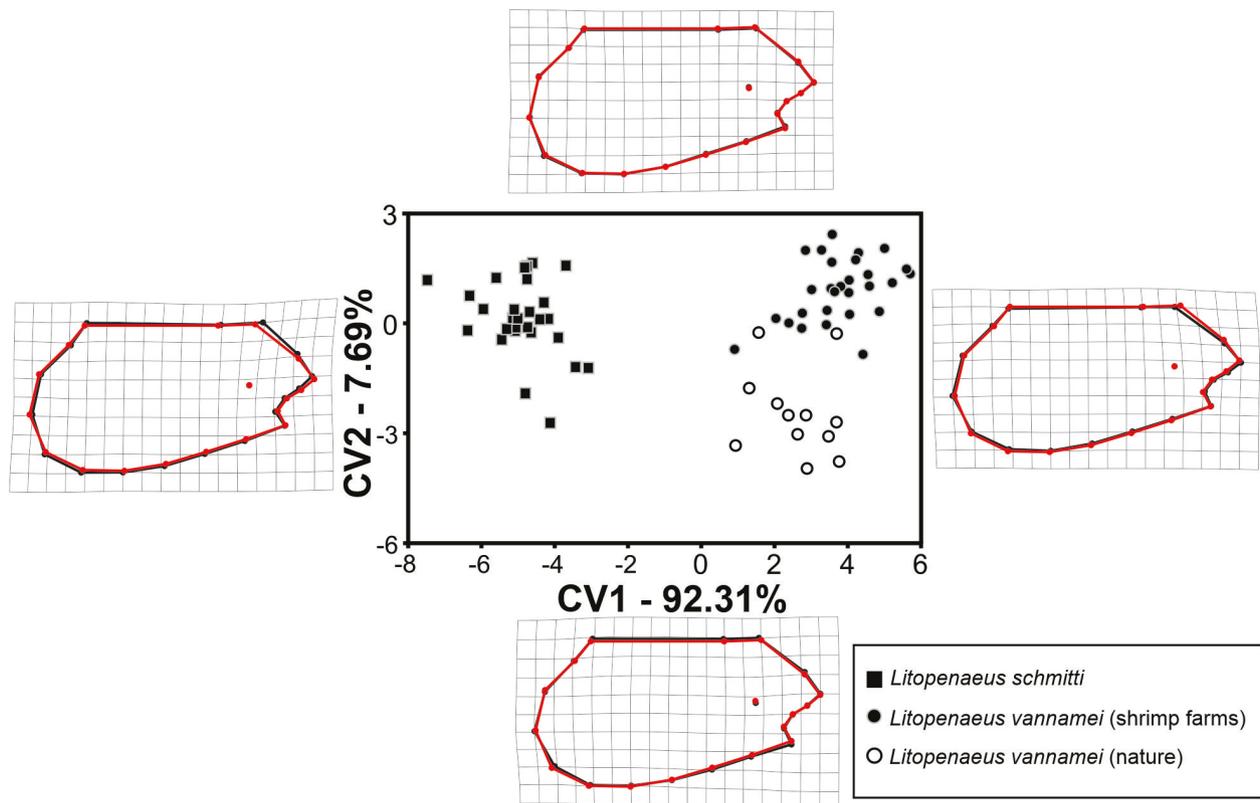


Figure 2. Canonical variable analysis of the 'species' (*Litopenaeus schmitti* and *Litopenaeus vannamei*) vs. 'origin' (nature and shrimp farms) factors as grouping factors and the deformation grids and shape wireframes for each investigated component. Red = *L. schmitti*; Black = *L.s vannamei*.

schmitti, and 66.66% for *L. vannamei* from the natural environment, and 96.42% for *L. schmitti* and 92.82% for *L. vannamei* from shrimp farms. There was no statistical difference between *L. vannamei* (procrustes distance = 0.0112; $P = 0.206$).

The two species of shrimp differ concerning the cephalothorax geometry, mainly in the rostrum stretch and relative position of the first rostral tooth to the hepatic spine. The cephalothorax shape of *L. schmitti* has a more stretched, dorsoventrally, and more compact profile (lower antero-posteral stretch), with points that form the base of the rostral spine closer (Landmark 6 and 7) (Fig. 3a). Additionally, the anatomical landmark representing the first rostral tooth (Landmark 7) revealed a vector displacement to the opposite side of the tip position of the hepatic spine (Fig. 4a). In contrast, *L. vannamei* presented a wider geometry and less stretched dorsoventrally, with the points of the base of the rostrum more distant from each other (Fig. 3a). Moreover, the point that represents the first rostral tooth showed an opposite displacement in relation to the anterior species, tending to a parallel position the tip of the hepatic spine (Fig. 4b).

DISCUSSION

Studies on the shape of cephalothorax in shrimps are recent and mainly deal with geometric morphometrics as a tool for assessing sexual dimorphism (Accioly et al. 2013, Sganga et al. 2016) or population discrimination (Bissaro et al. 2013, Torres et al. 2014, Zimmerman et al. 2011, Moraes et al. 2020). All previous studies were intra-specific, with no objective to examine the relationship between similar species or congeners. Although interspecific differences in the shape have already been observed in sister species of crustaceans (Giri & Loy 2008, Riedlecker et al. 2009), the present study is the first to investigate the body shape using geometric morphometrics technique by anatomical landmarks as a tool to aid the taxonomic discrimination among shrimp species.

Among the species used in this study, it was possible to observe a significant geometric variation between the cephalothorax of the native species *L. schmitti* and the non-native *L. vannamei*. Phylogenetic studies on shrimp of the genus *Penaeus sensu lato* Fabricius, 1798 argue that a single lineage of species of *Penaeus* s. l. colonized the Americas, recently, from the Pacific Western, and then they diverged in the lineages of *Farfantepenaeus* and *Litopenaeus*,

Table II. Statistical results for comparison of shape variation between grouping factors 'species' (*L. schmitti* and *L. vannamei*) and 'origin' (nature and shrimp farms).

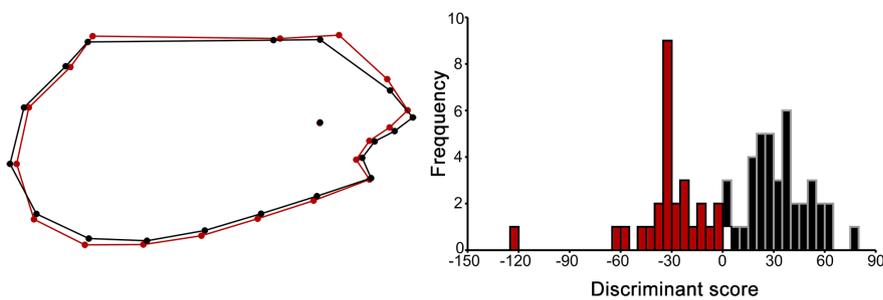
DFA	T ²	T ² P	D ²	Proc Dist	P ^{**}
<i>L. schmitti</i> x <i>L. vannamei</i>	1090.61	<0.01	8.13	0.0293	<0,01
<i>L. schmitti</i> (nature) x <i>L. vannamei</i> (nature)	1322.15	0.03	12.54	0.0273	<0,01
<i>L. schmitti</i> (nature) x <i>L. vannamei</i> (shrimp farms)	1227.37	<0.01	9.36	0.0308	<0,01
<i>L. vannamei</i> (nature) x <i>L. vannamei</i> (shrimp farms)	545.47	0.20	8.05	0.0112	0,31

DFA, Discriminant Function Analysis; T², Hotelling t test; D², Mahalanobis distance; Proc Dist, Procrustes distance. ** Significance value for the permeation test of the distance of procrustes between the groups.

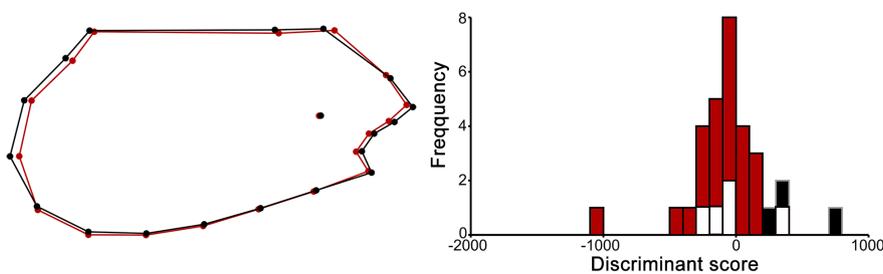
thus distributing to both sides of the Americas (Lavery et al. 2004). After the complete closure of the Isthmus of Panama, vicariant speciation occurred, leading to the different lineages currently found on each side of the Americas (Lavery et al. 2004, Maggioni et al. 2001). Though *L. schmitti* and *L. vannamei* present significant genetic divergences that are characterized as distinct species, they still share similarities in the general appearance (characteristic typical to the entire *Penaeus* s. l. group). Despite this similarity, differences in food, food conversion, growth rate, use of microhabitat, competition, and environmental conditions can be reflected in small differences in the shape of organisms

(Peres-Neto 1995). For example, the muscle growth pattern in shrimps is directly related to the type of diet and chemical composition (Mondal et al. 2014). Similarly, the conditions and habitat use directly influence the development of organisms and result in morphological and physiological changes (Bissaro et al. 2013, Castilho et al. 2007). These observations are frequent in species with a broad geographic distribution (Bissaro et al. 2013). Since *L. vannamei* and *L. schmitti* evolved in distinct marine ecoregions (*sensu* Spalding et al. 2007), the pattern observed in this study suggests that cephalothorax, and/or organs and internal musculature related to this structure, in *L. schmitti* was subjected to

a) *Litopenaeus schmitti* vs *Litopenaeus vannamei*



b) *Litopenaeus schmitti* vs *Litopenaeus vannamei* (nature)



c) *Litopenaeus schmitti* vs *Litopenaeus vannamei* (shrimp farm)

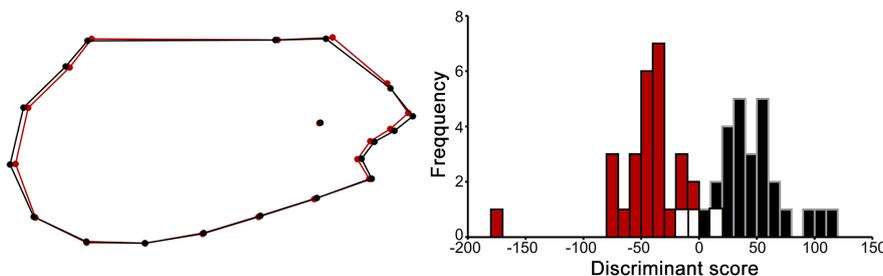


Figure 3. Comparison wireframe of cephalothorax shape and frequencies of discriminant function scores. Deviations between the corresponding anatomical landmarks between the analysis groups represent the vector displacement. Red = *Litopenaeus schmitti*; Black = *Litopenaeus vannamei*; White = frequency overlap.

distinct selective pressures from those that affected the evolutionary history of *L. vannamei*. Although this observation seems evident from the evolutionary point of view, it is not valid for all structures and all Crustacea taxa. Rosenberg (2002), for example, evaluated the shape of the largest chelipeds among species of the genus *Uca* and concluded what they called “unexpected result” that, although specific clades appear to present shape of distinct chelipeds, the analysis indicated a phylogenetic convergence (phylogenetic agglomeration) of the shape of the chelipeds in the whole genus.

For the adult male shrimps of the present study, our result evidenced a characteristic that can help in the macroscopic distinction between these two species of Penaeidae: the position of the base of the first rostral tooth, in relation to the tip of the hepatic spine. In *L. schmitti*, the first tooth of the rostrum develops in a position more anterior than in *L. vannamei*,

never aligning the tip of the hepatic spine. In *L. vannamei*, the first rostral tooth develops closer or in the same vertical plane in which the tip of the hepatic spine is visualized (Fig. 4). This shape characteristic, together with the observation of the structures of the petasma (Pérez-Farfante 1969, A.B. Moraes et al., unpublished data), can ensure field identification at the species level for these congeners.

Studies such as Bissaro et al. (2013), Torres et al. (2014) and Zimmerman et al. (2011) demonstrated that the physical structure of the environment, the flow of water, magnitude of the currents, and type of sediment could directly influence the shape of the cephalothorax of shrimps. It occurs because this structure is directly related to many functions (e.g., nutrition, locomotion, and perception), causing it to have different responses according to different environments (Zimmerman et al. 2011). In the case of *L. vannamei*, shrimp farms environments have very different physical and hydrological factors than what is found in the natural environment, which can cause differentiations in shape between the cultivated and natural environment groups. Although the results obtained do not indicate a statistically significant difference in shape ($P > 0.05$), it was possible to observe a slight distinction of shape in terms of prawn origin. This small dissimilarity between the origin of *L. vannamei* becomes more apparent when the non-native species is compared with the native *L. schmitti*. However, investigations with a new configuration of anatomical landmarks, including more detail in the capture of the carapace can help corroborate our current proposition.

Juvenile individuals of *Litopenaeus*, in general, have similar cephalothorax morphology (Pérez-Farfante 1969). Therefore, species of the same genus that co-occur can make it difficult to identify specimens at the species level. Our

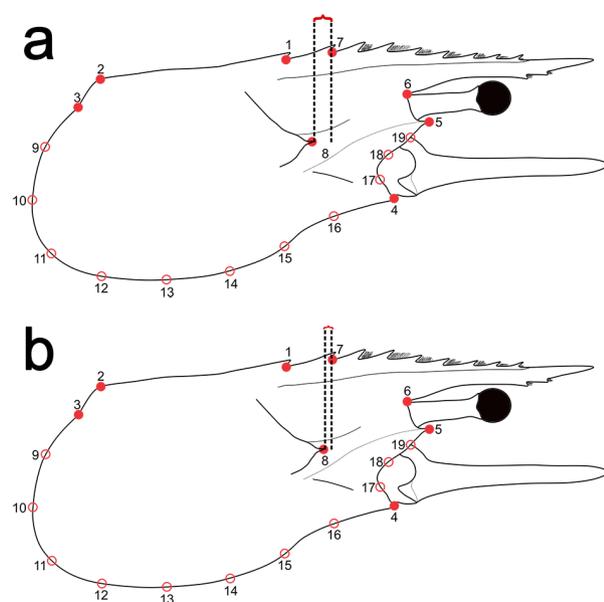


Figure 4. Cephalothorax right profile of *Litopenaeus* illustrating the difference in the position of the base of the first rostral tooth in relation to the tip of the hepatic spine between *Litopenaeus schmitti* (a) and *Litopenaeus vannamei* (b).

results show that congenital adults have a higher similarity of cephalothorax when *L. vannamei* is of natural capture. Until now, the characters that remain most reliable for the identification of juveniles of the genus are the structure of the petasma and the thelycum (Pérez-Farfante 1970, A.B. Moraes et al., unpublished data). We suggest that additional investigations using geometric morphometrics by anatomical landmarks can reveal whether the characteristics observed here for adult males can also be used to identify females and juveniles of both species. Additionally, linear measurements can be applied to quantify the difference in the position of the hepatic spine among the species. The authors also suggested the need for a comparative study between wild (from their geographic area of origin) and cultivated *L. vannamei*. This comparison would aim to evaluate whether genetic erosion resulting from consanguinity and low genotypic diversity, which occurs in cultivated animals (Doyle 2016), affects the shape of body structures and to quantify the extent of these changes.

In general, the study demonstrated the differences in shape, which can be the result of evolutionary influences. Since the species are related to biological invasions, this contribution can reduce the probability of identification errors that would lead to errors in the database on the history of invasion. Moreover, the description of the variation is also the first step in understanding the evolution of a complex morphological structure subjected to distinct evolutionary histories. The next step is to study how these variations are specifically related to the differences in the function of structures associated with cephalothorax.

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