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Date: 08-06-2016
To: "Agustín Garese" agustingarese@gmail.com
From: "Andreas Schmidt-Rhaesa" andreas.schmidt-rhaesa@uni-hamburg.de
Subject: ZOMO: Your manuscript entitled Biometry of sea anemone and corallimorpharian cnidae: statistical distribution and suitable tools for analysis

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Ref.: Ms. No. ZOMO-D-16-00025R1
Biometry of sea anemone and corallimorpharian cnidae: statistical distribution and suitable tools for analysis
Zoomorphology

Dear Dr. Garese,

I am pleased to tell you that your work has now been accepted for publication in Zoomorphology.

Thank you for submitting your work to this journal.

With kind regards

Andreas Schmidt-Rhaesa
Editor-in-Chief
Zoomorphology
COMMENTS TO THE AUTHOR:

Dear Dr. Garese,
many thanks for the careful revisions on your manuscript and their explanation. The manuscript is now fully accepted. We thank you for your contribution to Zoomorphology. Please expecte the proofs soon. Immediately after this, the article will be published online and a while later (depending on other manuscripts in line) in the print edition.
With best wishes,
Andreas Schmidt-Rhaesa
Chief Editor

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Zoomorphology

Biometry of sea anemone and corallimorpharian cnidae: statistical distribution and suitable tools for analysis

--Manuscript Draft--

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Abstract:	Many studies have dealt with data on the sizes of cnidae within different groups of anthozoans, such as Actiniaria, Corallimorpharia and Zoantharia. Statistical treatments of these data have been variable, according to the evaluation of the normality; hence the use of parametric or non-parametric tests. The normality of cnidocyst size data was assumed or proved by some authors; who used parametric tests to make comparisons. Other authors carried out non-parametric tests, or even proposed alternative analytical methods, such as the use of generalized linear models. Despite controversy about the statistical distribution of cnidae sizes, there has never been an attempt to study the normality of cnidocyst size data involving a significant volume of samples, using several specimens from various different species and using the same statistical approach. The objective of this paper is to evaluate statistical adjustment to a normal distribution of cnidocyst length from four sea anemone and one corallimorpharian species. The cnidoms of all species are detailed and the hypothesis of no intra-specific variation of cnidae sizes tested as a study case. Normality was accepted in 36.42% (sd = 17.91) of all data sets of all cnidocyst types analyzed from all studied species, while for the rest it was rejected. The evidence suggests that both normal and non-normal data sets are possible, although non-normality is slightly more frequent. Intra-specific variation of cnidocyst sizes is shown in 96.82% of the analyzed data sets. This paper provides a simple and detailed methodology to perform comparisons of cnidae size data.

Response to the Reviewers' Comments

We have considered and included all corrections of the reviewers. As you can see we modified the title as was suggested by the revisor 2. Also the English version of the paper was read and improved by a native English speakear biologist (Dr. Charles Griffiths, University of Cape Town, South Arfrica). In the discussion we added a phrase about why the cnidae are not regularly distributed, including some new references, according to the suggested by reviewer 1.

Biometry of sea anemone and corallimorpharian cnidae: statistical distribution and suitable tools for analysis

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Abstract

Many studies have dealt with data on the sizes of cnidae within different groups of anthozoans, such as Actiniaria, Corallimorpharia and Zoantharia. Statistical treatments of these data have been variable, according to the evaluation of the normality; hence the use of parametric or non-parametric tests. The normality of cnidocyst size data was assumed or proved by some authors; who used parametric tests to make comparisons. Other authors carried out non-parametric tests, or even proposed alternative analytical methods, such as the use of generalized linear models. Despite controversy about the statistical distribution of cnidae sizes, there has never been an attempt to study the normality of cnidocyst size data involving a significant volume of samples, using several specimens from various different species and using the same statistical approach. The objective of this paper is to evaluate statistical adjustment to a normal distribution of cnidocyst length from four sea anemone and one corallimorpharian species. The cnidoms of all species are detailed and the hypothesis of no intra-specific variation of cnidae sizes tested as a study case. Normality was accepted in 36.42% (sd = 17.91) of all data sets of all cnidocyst types analyzed from all studied species, while for the rest it was rejected. The evidence suggests that both normal and non-normal data sets are possible, although non-normality is slightly more frequent. Intra-specific variation of cnidocyst sizes is shown in 96.82% of the analyzed data sets. This paper provides a simple and detailed methodology to perform comparisons of cnidae size data.

Keywords

Actiniaria, Corallimorpharia, Cnidom, Normal Distribution, Generalized Linear Models.

Introduction

Cnidocysts are a diagnostic character of the phylum Cnidaria. They consist of an intracellular secreted capsule with a coiled filament inside that is extruded in response to a stimulus. Cnidocysts are classified into three categories: nematocysts, spirocysts, and ptychocysts. Nematocysts are present in all members of the phylum and are the most variable category, with 25 recognized morphologic types. Spirocysts are restricted to hexacorallians of the class Anthozoa, whereas ptychocysts are solely present in the subclass Ceriantharia (Mariscal 1974, 1984; Fautin & Mariscal 1991); both are morphologically unique types. Each cnidarian species has a determinate assemblage of cnidocysts, called the cnidom (Weill 1926). In the order Actiniaria (sea anemones *sensu stricto*), it is generally established that taxonomic studies should always include a description of the species' cnidoms, as suggested by Carlgren (1900). Also, details of the size ranges of each cnidocyst present in all structures of a sea anemone should be provided, as proposed by Hand (1955a, b; 1956). Despite this standard procedure, the taxonomic value of cnidocysts is relative and variable, some families or genera of sea anemones can be characterized by the composition of cnidocysts (Fautin 1988, 2009). Moreover, the value of cnidocyst characteristics at species level is weaker, mainly due to the usual intraspecific variation of cnidae sizes (Allcock et al. 1998; Watts et al. 2000; Ardelean and Fautin 2004; Francis 2004; Acuña et al. 2003, 2004, 2007; Ryland et al. 2004), and in these situations the cnidome should be used to complement other characters in arriving at an identification (Acuña et al. 2003).

Several studies have deal with biometrics data of cnidae in different groups of anthozoans, such as Actiniaria, Corallimorpharia and Zoanthidea (Thomason 1988, Williams 1996, 1998, 2000; Chintiroglou et al. 1997; Allcock et al. 1998; Acuña et al. 2004, 2007; Ardelean and Fautin 2004; Ryland et al. 2004; Francis 2004; Acuña and Garese 2009). Statistical treatments of this kind of data have been variable according to the evaluation of their normality, and acceptance or rejection of it. Some authors proved normal distribution of sizes of cnidocysts, either from raw data or transformed data, and consequently used parametric tests to make comparisons (Williams 1996, 1998, 2000; Allcock et al. 1998; Watts et al. 2000; Ardelean and Fautin 2004; Francis 2004; Ryland et al. 2004). By contrast, other authors directly applied non-parametric test (Chintiroglou et al. 1997), or did so before refusing normality of the data (Acuña et al. 2003). Furthermore, more recent studies proposed alternative analysis, more powerful than non-parametric ones, such as the implementation of generalized linear models (GLM) (Acuña et al. 2004, 2007; Acuña and Garese 2009). Williams (1996, 1998, 2000) focuses specifically on statistical methods to compare cnidae size on sea anemones and concluded that actiniarian cnidocyst data follow a normal (Gaussian) distribution. Also Williams (1998), proposed a protocol of

analysis which has been followed by other authors (Allcock et al. 1998; Watts et al. 2000; Ardelean and Fautin 2004). Ardelean and Fautin (2004) analyzed the cnidom in one specimen of *Actinodendron arboretum* (Quoy and Gaimard 1833), even though representativeness of their sample is weak and this limits the value of their finding. In this study a few cases normal distribution was rejected; but it was proved in the majority of data. In the same vein, no departures from normality were observed by Allcock et al. (1998) in two morphs of *Actinia equina* (10 specimens each) and Watts et al. (2000), who compared nematocysts of *A. equina* (eight specimens) and *Actinia prasina* (eight specimens). Ryland et al. (2004) use a robust statistical approach to study cnidocysts from the zoanthid species *Acrozoanthus australiae*, and even though they found that for 12% of samples normal distribution was rejected, they noted there was no clear evidence of departure from normality. On the other hand, Acuña et al. (2003) studied nematocyst sizes from acontia of *Tricnidactis errans* Pires, 1988, *Anthothoe chilensis* Lesson, 1830 and *Haliplanella lineata* (Verrill 1869) (five specimens each), and refuted Williams about the normality of cnidocyst length data, at least from those acontiarians sea anemones; recommending testing normality previous to any biometric study of cnidae. Acuña et al. (2004, 2007), based on the case where non-normal distribution was demonstrated (Acuña et al. 2003), introduced a novel analysis of cnida sizes using generalized linear models (GLM) to produce comparisons. Furthermore, Acuña and Garese (2009) analyzed the cnidom from acrospheres of the corallimorpharian *Corynactis carnea* and found that only one of six types of cnidocysts, the spirocysts, follows a normal distribution. The conflicting results of these studies highlight the need for clarification concerning the normal or non-normal distribution of cnidae sizes. Despite the controversy, a study of the normality of cnidocyst sizes data with a representative volume of samples and analyzed with the same statistical approach, has never been done. This approach could improve knowledge concerning of statistical distribution of cnidocyst length data, allowing more robust conclusions that ensure the use of appropriate methods to deal with these data. The main purpose of this study was thus to evaluate the statistical adjustment of the length of cnidocysts to normal Gaussian distribution from four sea anemones and a corallimorpharian species, detailing and analyzing their complete cnidoms. Moreover, the hypothesis of no intraspecific variation of cnidae sizes in the five species was tested as study cases, with the objective of proposing a procedure to deal with this kind of data in comparisons or variation analysis.

Materials and Methods

The complete cnidoms of the following five species were studied: *Aulactinia marplatensis*; *Bunodosoma zamponii*, and *Bunodactis octoradiata* (Family Actiniidae: Actiniodea: Actiniaria), *Anthothoe chilensis* (Family

Sagartiidae: Metridioidea: Actiniaria), and *Corynactis carnea* (Family Corallimorphidae: Corallimorpharia). In this way, different taxonomic groups of sea anemones *sensu lato* are represented. Thus, a wide variety of cnidocysts was sampled, which resulted in wider and well-supported conclusions.

Sampling of sea anemones

Corynactis carnea were collected during a survey by the vessel "Oca Balda" (10/9/88) (INIDEP, expedition 04-88). The collecting coordinates were 38º11'S - 57º03'W, the depth 59m, the temperature 10.3ºC, and the salinity 33.7‰. *Bunodactis octoradiata* were sampled (15/12/09) by hand from the intertidal zone of Punta Cueva (49°13'10"S, 67°40'20"W), Puerto San Julián, Santa Cruz Province, Argentina.

The remaining species (*A. marplatensis*, *B. zamponii*, *A. chilensis*) were all collected (09/02/11, 14/05/12, 14/05/12, respectively) by hand during low tides on the rocky shore intertidal of Punta Cantera (38º04'S–57º32'O), Mar del Plata, Buenos Aires, Argentina. All samples were fixed in 5% formaldehyde and subsequently in ethanol.

Cnidocysts sampling

The complete cnidom (from tentacles, column, mesenterial filaments and actinopharynx; and acrorhagi and acontia in those species that possess them) was analyzed in 12 specimens of each species, with the exception of *B. octoradiata* for which 10 specimens were studied. Cnidocysts were identified following England (1991). Statistical analyzes were carried out beginning with length data from 30 intact and unfired cnidocysts of each type present in all structures of each studied species. In some cases, it was not possible to reach 30 measures of capsules due to their very low abundance; in these cases all cnidocysts found were measured. The sampling was made by mean of squashes, and a Zeiss Axiolab microscope with oil immersion at 1000x magnification was employed for this purpose. In total, 27 224 measurements were obtained.

Biometry

Complete cnidoms of the five studied species were detailed, by identifying all types present in the different structures of the sea anemones. Moreover, descriptive statistical parameters of their sizes (length and width), such as mean, standard deviation, minimum and maximum, and abundances, were calculated.

Normality of length data of all types of cnidocysts was tested by mean of Shapiro-Wilks test ($\alpha = 0.05$) over residuals of a linear normal model, except those types where was impossible to achieve a representative set of at

least 100 data among all individuals. Then, fit to normal model was evaluated graphically with QQ-plots (Standardized residuals vs. Theorical quantiles) and dispersion diagrams (residuals vs. fitted values). Afterward, in cases where normality was accepted, an ANOVA was conducted to evaluate differences between individuals for each cnidocyst into species. On the other hand, when length data were not normally distributed, a Generalized Linear Model (GLM) with only one categorical covariate (individuals) was carried out. This GLM can be seen as a one-factor analysis of deviance, in accordance with Acuña et al. (2004). In that sense, a GLM with gamma distribution of errors and inverse as link function was fitted, taking the model as follows:

$$g(\text{length}) = \beta_0 + \beta_1(\text{individual}) + \varepsilon$$

Fit to gamma distribution was explored using graphics of QQ-plots (standardized residuals vs. GLM theorical quantiles) and dispersion diagrams (residuals vs. GLM fitted values), identical to what was done for normal distribution cases. Then, a t-test for coefficients of the model (β_1) was performed to test the hypothesis of equal mean of length of each cnidocyst for all species. All statistical analyses were performed with R program (R 2008).

Results

Corynactis carnea

The cnidom of *C. carnea* (Fig. 1, Electronic Supplementary Material) included only spirocysts in the tentacles. Five types of cnidocysts were found in the column and were identified as two size ranges of microbasic p-mastigophores, two microbasic b-mastigophores, and holotrichs. Mesenterial filaments contained microbasic p-mastigophores and two types of holotrichs, while the actinopharynx featured holotrichs and microbasic b-mastigophores. The sizes of cnidae found in *C. carnea* are detailed in Table 1. The cnidom of this species is complete, with cnidocysts from acrospheres, which were studied by Acuña and Garese (2009). P-values for Shapiro-Wilks normality test are shown in Table 1, the normality was accepted in 5 of 9 analyzed data sets for the species [Tentacles: spirocyst; Column: holotrich; Mesenterial filaments: holotrich I, holotrich II; Actinopharynx: holotrich]; while data of microbasic p-mastigophores I from the column were not considered, due to low N achieved. Adjustment of data to a normal linear model and to a generalized linear model, when the normality was rejected, was explored graphically (Figs. 2, 3, 4, 5 Electronic Supplementary Material). The fit to the GLM was appreciably better than to the normal model in all applied cases (Figs. 2, 4 Electronic Supplementary Material). This best fit is evidenced with residuals in closer scales in those cases, between 15 and 25 times smaller (Figs. 3, 5, Electronic Supplementary Material).

ANOVA tests show significant differences between individuals in all analyzed types [Tentacles: spirocyst ($F_{11, 348} = 4.41$, $p < 0.001$), Column: holotrich ($F_{11,307} = 2.87$, $p = 0.001$), Mesenterial filaments: holotrich I ($F = 10.12$, $p < 0.001$) y holotrich II ($F = 18.73$, $p < 0.001$), Actinopharynx: holotrich ($F = 10.85$, $p < 0.001$)]. Furthermore, cnidocyst types with no normal length of the capsules exhibited statistically significant differences between individuals according to the t-test for the coefficients (β_1) of the GLM applied (Table 2, Electronic Supplementary Material).

Anthothoe chilensis

Cnidocysts present in tentacles of *Anthothoe chilensis* (Fig. 6, Electronic Supplementary Material) were basitrichs, spirocysts, mesobasic p-mastigophores and haplonemes (probably atrichs, according to Excoffon et al. 1997); three cnidocyst types were observed in the column: basitrichs, microbasic p-mastigophores, and haplonemes (atrichs). Moreover, this species had basitrichs, two types of microbasic p-mastigophores and mesobasic p-mastigophores in mesenterial filaments; while in the actinopharynx the greatest diversity of cnidocysts was observed, with six types, two basitrichs, microbasic b-mastigophores, two microbasic p-mastigophores and mesobasic p-mastigophores. Finally, the cnidom was completed with basitrichs and mesobasic p-mastigophores in acontia, the characteristic structure of all species within the superfamily Metridiodea. Table 3 shows size ranges of all cnidocysts and p-values for normality test. Of all the data sets analyzed for this species, in 13 normality was rejected, while two types of cnidocyst fitted to a normal distribution [Actinopharynx: microbasic p-mastigophore II; Acontia: basitrich]. Apart from that, four types of cnidocyst were not considered, because they did not achieve 100 data in total (Table 3). In that sense, no normality of cnidae sizes is the rule in this species, with 13 of 15 data sets studied. Figs. 7 and 8 (Electronic Supplementary Material) show graphically the fit to a normal linear model of the lengths of all cnidocysts of *A. chilensis*. When the normality was not accepted the GLM was applied and its fit to the data can be observed graphically in Figs. 9 and 10 (Electronic Supplementary Material). In general, all QQ-plots showed an acceptable fit of the GLM; however in some types of cnidocysts, the adjustment decreased at lower and upper values (Fig. 9 a, c, g, h, m, Electronic Supplementary Material). Despite that, a better fit was observed to the GLM than the normal linear model; that is evidenced by magnitudes of residuals being 15 - 35 times lower (Figs. 8, 10, Electronic Supplementary Material). In terms of intraspecific variation of cnidae size, significant differences between individuals for microbasic p-mastigophores II from actinopharynx and basitrichs from acontia were evidenced as results of ANOVA ($F_{11,184} = 3.89$, $p < 0.001$; $F_{11,348} = 6.22$, $p < 0.001$, respectively).

Moreover, t-tests for the GLM β_1 coefficients also showed statistical differences in the cnidae size among individuals for all the cnidocysts analyzed (Table 4, Electronic Supplementary Material).

Bunodosoma zamponii

This species possessed a cnidom (Fig. 11, Electronic Supplementary Material) with basitrichs and spirocysts in the tentacles and these same types, plus holotrichs present in acrorhagi. The column held the most diverse cnidom with five types of cnidocysts identified, two types of basitrichs, spirocysts and two types of holotrichs. In addition, three types of microbasic b-mastigophores, and microbasic p-mastigophore were found in mesenterial filaments. Lastly, the actinopharynx had four types: basitrichs, spirocysts, microbasic b-mastigophores and microbasic p-mastigophores. Sizes of all cnidocyst are summarized in Table 5, also the p-values of normality test for each of them. This analysis was performed for all cnidocyst except for the microbasic p-mastigophore of actinopharynx, due to low N achieved. Seven out of the 17 data set studied fitted to a normal distribution [Tentacles: basitrich; Actinopharynx: holotrich; Column: basitrich II, spirocyst; Mesenterial filaments: microbasic p-mastigophore; Actinopharynx: spirocyst microbasic b-mastigophore], while for the remaining types normality was rejected (Table 5). Figs. 12 and 13 (Electronic Supplementary Material) explore how the linear normal model fits the different cnidocysts by mean of QQ-Plots and dispersion graphics of residuals versus fitted values, respectively.

In cases when the normality was rejected, the data had a good fit to the GLM (Fig. 14, Electronic Supplementary Material). However, an exception could be noted in the holorich I from the column, which presented a poor fit in the upper values (Fig. 14e, Electronic Supplementary Material). Nevertheless, that behavior was also observed in its fit to the normal linear model (Fig. 12e, Electronic Supplementary Material), so could correspond to particularities of that data set. Values of residuals of the GLM were on average around 25 times smaller than those of the linear normal model (Figs. 13, 15, Electronic Supplementary Material), in accordance with findings in *C. carnea* and *A. chilensis*.

The analysis carried out to test if there were an intraspecific variation of cnidocyst sizes showed that 15 out of 17 analyzed cnidocysts had statistically significant differences, which were evidenced by ANOVA [Tentacles: basitrich ($F_{11,345} = 35.67$, $p < 0.001$); Acrorhagi: holotrich ($F_{11,347} = 61.15$, $p < 0.001$); Column: basitrich II ($F_{11,348} = 6.07$, p value <0.001); Mesenterial filaments: microbasic p-mastigophore ($F_{11,340} = 16.64$, $p < 0.001$); Actinopharynx: microbasic b-mastigophore ($F_{11,348} = 6.83$, $p < 0.001$)] or t-test for the coefficient of the GLM (Table 6, Electronic Supplementary Material). On the other hand, there were no statistical differences

between individuals in the spirocysts from two different structures, column ($F_{11,109}$ = 1.54, p = 0.12) and actinopharynx ($F_{9,107}$ = 1.62, p = 0.11).

Aulactinia marplatensis

This species presented in its cnidom (Fig. 16, Electronic Supplementary Material) two types of cnidocysts in the tentacles, spirocysts, and basitrichs; in column showed identical cnidocysts to the tentacles, although with two size types of basitrichs, totaling three types. Mesenterial filaments had the greatest diversity of cnidae in *A. marplatensis*, with six types: spirocysts, two types of basitrichs, microbasic b-mastigophores, holotrichs and microbasic p-mastigophores. The actinopharynx possessed three different cnidocysts: spirocysts, basitrichs, and microbasic amastigophores. Details about range sizes of all cnidocysts of *A. marplatensis* and p-values of normality test can be seen in Table 7. Analysis of fit to a normal distribution revealed that in seven cnidae types the normality was accepted [Tentacles: spirocyst, basitrich; Actinopharynx: spirocyst, basitrichs I and II; Mesenterial filaments: basitrich II, microbasic b-mastigophore], while this was rejected in the others seven cases. Spirocyst and holotrich of mesenterial filaments were not studied due to low N found. Figs. 17 and 18 (Electronic Supplementary Material) explore the adjustment of the normal linear model to the data of each cnida size. In cases where cnidocysts did not follow normal distribution, the fit of the GLM was evaluated and, as shown in Figures 19 and 20 (Electronic Supplementary Material), the adjustment to this model improved that observed for the normal linear model. Then, comparing Figs. 18 and 20 (Electronic Supplementary Material) revealed reductions of magnitudes of residuals in the GLM with respect to the normal model on a scale approximately 25 times lower.

Comparisons of cnidae sizes between individuals resulted in significant differences for all types according to the results of ANOVA [Tentacles: basitrich ($F_{11,\ 348}$ = 18.91, p < 0.001), spirocyst ($F_{11,345}$ = 18.74, p < 0.001); Mesenterial filaments: basitrich II ($F_{11,\ 348}$= 4.54, p < 0.001), microbasic b-mastigophore ($F_{11,347}$ = 12.92, p <0.001); Actinopharynx: basitrich I ($F_{11,346}$ = 13.45, p < 0.001), basitrich II ($F_{11,346}$ = 10.73, p < 0.001), spirocyst ($F_{11,300}$ = 12.32, p < 0.001)] or t-test for the β_1 of the GLM (Table 8, Electronic Supplementary Material).

Bunodactis octoradiata

The composition of cnidocysts of this species consisted basically of spirocysts, basitrichs, and microbasic p-mastigophores, making this the species with the lowest diversity of cnidae types studied. The tentacles

contained spirocysts and two size types of basitrichs, these last also shaped the cnidom of the column and mesenterial filaments though these basitrichs were accompanied by microbasic p-mastigophores in the inner structure. Finally the actinopharynx possessed a unique type of basitrich and microbasic p-mastigophores. Sizes of all cnidocysts and p values of normality test for residuals of a linear normal model are listed together in Table 9; while images of each cnida type were previously published in Garese et al. (2014). Normality was proved for two out of 10 analyzed cnidocysts [Tentacles: spirocyst; Mesenterial filaments: microbasic p-mastigophore]. The fit to a normal linear model was also graphically exploring for all cnidae (Figs. 21, 22, Electronic Supplementary Material). For the data sets with non-normal distribution, the GLM was applied and their fit was also graphically evaluated (Figs. 23, 24, Electronic Supplementary Material). A good adjustment of this generalized linear model was observed for the cnidocysts of *B. octoradiata* as well was reported in the remaining species of the present paper. Residuals of the GLM presented a range of distributions 20 - 35 times narrower than those of normal model (Figs. 22, 24, Electronic Supplementary Material).

Variation analyses of capsule length reflected significant differences between individuals for all cnidocysts [ANOVA: Tentacles: spirocysts ($F_{9,290} = 3.21$, $p < 0.001$); Mesenterial filaments: microbasic p-mastigophore ($F_{9,290} = 19.08$, $p < 0.001$); see also Table 10 (Electronic Supplementary Material) for p value of the t-test of β_1 of the GLM].

Normality by cnida type

Results showed that the normality in the studied species was accepted in different percentage of all data sets analyzed: 55% in *C. carnea*, 15.38 % in *A. chilensis*, 41.17% in *B. zamponi*, 50% in *A. marplatensis* and 20% in *B. octoradiata*. Thus, taking data sets of the fives species together, the mean of acceptance of normal distribution was 36.41% (sd = 17.91), suggesting that both acceptance and rejection of normality for cnidocyst size data are possible but no normality is slightly more probable. Otherwise, analyzing our results depending on cnida type, independently of structure or species, showed that spirocysts and holotrichs were the unique types where normality was accepted in more than 50% of data sets. Holotrich data achieved 83% of normality, whereas spirocyst data achieved 66%; it seems that capsule length data of these types tend to fit more frequently to a normal distribution. On the other hand, for the rest of cnidocysts, normal distribution was accepted in less than 33% of data sets. In microbasic p-mastigophore, normality acceptance was 33%, while 28% of data sets of basitrichs and microbasic b-mastigophore fitted to a normal distribution. Moreover, for mesobasic p-mastigophores, microbasic amastigophores and haplonemes all data sets were not normally distributed, however

in the last two only one data set was analyzed. These results suggest that even though both normal and non normal distribution is possible in any type of cnidocysts, certainly the holotrichs usually follow a normal distribution and the spirocysts too, but in less proportion.

Intraspecific variation

The study shows that 96.82% of the analyzed data set of cnidocyst sizes revealed significant differences between individuals. Only spirocysts from the column and actinopharynx of *B. zamponii* did not exhibite intraspecific variation.

Discussion

As mentioned by Williams (1996), the study of the statistical analyzes of cnidae sizes was surprisingly misleading by sea anemones specialists, despite routine use at least in taxonomic studies. Before this publication only Williams (1996, 1998, 2000) and Acuña et al. (2003, 2004, 2007, 2011) focused on statistical methods to deal with cnidae size comparisons, proposing some protocols or statistical tools. However, these authors arrived at different conclusions. Williams (1996, 1998) affirmed that data of cnidocyst sized followed a normal (Gaussian) distribution; Acuña et al. (2003) found that this was not true in several species of acontiarian sea anemones.

Williams (1996, 1998, 2000) carried out normality analysis in nine species and data from the majority of the species correspond to a single type of cnidocyst, mainly basitrich (although some spirocysts, microbasic amastigophore and p-mastigophores were also studied), from a particular tissue of one specimen. Although this limited sampling could limit the scope of Williams' conclusions, some authors have followed that protocol and did not generally find departures from normality (Allcock et al. 1998; Watts et al. 2000; Ardelean and Fautin 2004).

On the other hand, Acuña et al. (2003, 2004) based on non-normality demonstrated in some data sets of cnidocyst length implemented the use of generalized linear models to analyze this kind of data, which are parametric tools that do not follow a normal distribution. The scope of the results of Acuña et al. (2003, 2004) could be extensive to acontiarian sea anemones, but more generalizations may not be assessed. Then, in their work on *Oulactis muscosa*, Acuña et al. (2007) noted the normal distribution of cnidae size data is uncommon and hence should be rejected.

We agree partially with Williams (1996, 1998) and Acuña et al. (2003), because we found that both normal distribution and non-normal distributions of cnidae length data are possible, although non-normality is slightly more frequent. In accordance with Acuña et al. (2003) we observed cnidocysts non-normal distributed even in non-acontiarian sea anemones (i.e. *B. zamponii, B. octoradiata, A. marplatensis*); however, we also agree with the normality proposed by Williams (1996, 1998) finding it in around 40 % of our data sets. Moreover, in zoanthids, Ryland et al. (2004) showed departure from normality in 12% of their samples and pointed out that contradicted the results of Williams (1996, 1998).

The present paper brings to light the normal or non-normal distribution of cnidocyst length and provides compelling evidence about this matter, supported by a representative sampling. According to our results, there is evidence to indicate that both normality and non-normality are possible in different data sets of a species; and the proportion of them could vary in different ways, depending on taxa of study or, in particular cases, type of cnidocyst. For example, based on our results in *A. chilensis*, acontiarian sea anemones may have a low percentage of acceptance of normality in cnidae size, which is coincident with those of Acuña et al. (2003). Meanwhile, the analysis in actinoidean sea anemones (*B. zamponii, B. octoradiata, A. marplatensis*) indicated that both normality and no normality could be equally probable; nevertheless in *B. octoradiata* acceptance was achieved in only 20% of the data sets. In the corallimorpharian *C. carnea* results reflect a similar situation of Actinoidea, with an acceptance of normality of around 50%, however if we consider the results of Acuña and Garese (2009) adding the cnidom of acrospheres of the species, the percentage of acceptance drops to 40%. As for cnidocyst types, we found a pattern of higher acceptance of normality in holotrichs and spirocysts (80% and 60% respectively), suggesting that in these cases the normal distribution of length data is more frequent and in opposite way for other cnidocysts. Different hypothesis have been proposed to explain the high variability of cnidocyst size (e.g. Robson 1988; Zamponi and Acuña 1991; Karalis and Chintiroglou 1997; Francis 2004). This not regular distribution of some cnidocysts could be also attributed to a particular pattern of cnidogenesis even in different tissues from the same species.

Thus, normality must be tested in any biometric study of cnidocyst for use the more appropriated statistical tools. In that sense, Williams (1996, 1998) proposed a protocol where the normality is tested and secondly, if it is rejected, data should be transformed to achieve normality or, if that does not happen, non-parametric tests must be employed. This author used a particular statistical test employing tools of a specific statistical software (MINITAB), where starting from raw data, normal scores (a command of MINITAB) are calculated based on standard normal distribution, and finally a correlation test is carried out between the raw data

and the normal scores. Acuña et al. (2004) introduced the use of generalized linear models (GLMs) with gamma errors to be implemented in data set with non-normal distribution, being a parametric tool they are more powerful than non-parametric tests, and also do not force the data by mean of transformations to unusual scales (Hastic and Tibshirani 1990). The present paper provides a simple and clear methodology to perform comparisons of cnidae size data. This methodology consists of the evaluation of normality of residuals of a normal linear model as of raw data, then if the normality is accepted, ANOVA is used, and if it is rejected a GLM with gamma distribution for errors is fitted to make a t-test for the coefficient of the model. This way removes the need for transformation of data, the use of non-parametric tests and avoids dependence on particular statistical software; Figure 1 shows a graphical resume of the treatment for cnidae length data carried out in this work, the proposed methodology can be made with the free program R (R 2008), or any statistical software.

Otherwise, our study showed that the intra-specific variation of cnidae size is a fact in sea anemones. According to our results, percentage of rejection of our hypothesis is achieved in almost 97% of all data sets; these results are in good agreement with previous works (Williams 1996, 1998; Allcock et al. 1998; Watts et al. 2000; Ardelean and Fautin 2004; Francis 2004; Acuña et al. 2003, 2004, 2007; Ryland et al. 2004; Acuña and Garese 2009). Although the intra-specific variation suggests decreasing taxonomic value of cnidocysts, their study is still useful in other kinds of research, such as comparisons between different populations of the same species (Acuña and Zamponi 1997), differentiation of morphotypes of a species (Allock et al 1998; Watts et al. 2000; Gonzále-Muñoz pers. com), or to establish more precise differences among closely-related species (Watts et al. 2000; Martínez-Beraldés et al. 2014). Internal variation in a data set does not preclude a comparison with another data set because the variation between them could be different, however and hence these types of studies must be statistically well supported and suitable statistical tools should be used.

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Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

The authors declare that there is no ethical issue associated with their work. This study was performed on preserved specimens.

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Figure captions

Fig. 1 Diagram of the proposed methodology for the statistical analyzes of cnidae sizes data

Table 1 Cnidom of *Corynactis carnea*

Tissue	Cnida type	Range (min-max) length (mean±sd) x width (mean) [µm]	n	N	p value
Tentacle	Spirocyst	27-47 (36.84 ± 3.63) x 3-6 (3.7)	12/12	360	0.40
Column	Microbasic p-mastigophore I	34-53 (43.31 ± 3.29) x 8-15 (9.85)	7/12	86	▲
	Microbasic p-mastigophore II	17-36 (23.07 ± 3.66) x 4-10 (6.9)	12/12	200	<0.001*
	Microbasic b-mastigophore I	24-33 (27.27 ± 3.00) x 6-9 (7.4)	3/12	11	▲
	Microbasic b-mastigophore II	14-32 (19.36 ± 2.84) x 3-8 (4.79)	12/12	360	<0.001*
	Holotrich	40-60 (49.09 ± 3.77) x 9-20 (14.09)	12/12	319	0.83
Mesenterial filament	Microbasic p-mastigophore	19-49 (31.27 ± 6.42) x 5-14 (9.07)	12/12	360	0.006*
	Holotrich I	65-98 (83.68 ± 5.31) x 20-40 (32.29)	12/12	360	0.24
	Holotrich II	30-60 (43.54 ± 5.55) x 9-25 (14.89)	12/12	360	0.11
Actinopharynx	Microbasic b-mastigophore	19-37 (29.94 ± 2.91) x 3-6 (4.33)	12/12	360	<0.001*
	Holotrich	29-52 (41.3 ± 3.89) x 8-18 (12.92)	12/12	360	0.11
Acrosphere [a]	Spirocyst	22-80 (51 ± 10.63)	12/12	360	-
	Microbasic p-mastigophore I	20-55 (33.22 ± 4.52)	12/12	360	-
	Microbasic p-mastigophore II	43-88 (65.85 ± 6.44)	12/12	360	-
	Microbasic b-mastigophore I	31-70 (41.45 ± 4.05)	12/12	360	-
	Microbasic b-mastigophore II	30-86 (50.52 ± 7.79)	12/12	360	-
	Holotrich	54-98 (78.5 ± 7.08)	12/12	360	-

N total number of measured cnidocysts, *n* proportion of number of specimens in which each cnida was found. *Normality rejected. ▲ Analysis was not carried out due to the low N achieved.[a] Extracted from Acuña & Garese (2009)

Table 3 Cnidom of *Anthothoe chilensis*

Tissue	Cnida type	Range (min-max) length (mean±sd) x width (mean) [µm]	n	N	p value
Tentacle	Basitrich	15-29 (22.04 ± 2.85) x 2-4 (3.06)	12/12	360	0.005*
	Spirocyst	13-34 (21.32 ± 3.79) x 2-6 (3.48)	12/12	360	0.008*
	Mesobasic p-mastigophore	15-25 (21.87 ± 1.78) x 3-7 (4.37)	12/12	360	<0.001*
	Haploneme	11-18 (14.45 ± 1.54) x 3-4 (3.61)	8/12	42	▲
Column	Basitrich	8-20 (12.01 ± 2.34) x 2-3 (2.11)	12/12	360	<0.001*
	Microbasic p-mastigophore	13-21 (15.41 ± 1.29) x 3-5 (3.94)	12/12	360	0.004*
	Haploneme	13-22 (17.49 ± 1.53) x 3-5 (3.93)	12/12	360	0.002*
Mesenterial filament	Basitrich	11-23 (16.22 ± 1.62) x 2-3 (2.05)	12/12	360	<0.001*
	Microbasic p-mastigophore I	8-16 (10.75 ± 1.19) x 3-7 (4.80)	12/12	327	<0.001*
	Microbasic p-mastigophore II	14-24 (19.13 ± 2.14) x 4-7 (5.05)	12/12	360	<0.001*
	Mesobasic p-mastigophore	16-32 (26.22 ± 2.05) x 3-6 (4.31)	12/12	360	0.02*
Actinopharynx	Basitrich I	21-32 (26.26 ± 1.71) x 2-5 (3.19)	12/12	348	0.001*
	Basitrich II	10-18 (13.42 ± 1.68) x 2-3 (2.04)	11/12	99	▲
	Microbasic b-mastigophore	17-22 (19.36 ± 1.50) x 3-4 (3.18)	7/12	11	▲
	Microbasic p-mastigophore I	9-20 (16.33 ± 2.53) x 4-6 (4.90)	8/12	21	▲
	Microbasic p-mastigophore II	15-27 (19.27 ± 2.12) x 3-6 (4.51)	12/12	200	0.05
	Mesobasic p-mastigophore	16-29 (20.80 ± 2.01) x 3-7 (4.79)	12/12	352	0.002*
Acontia	Basitrich	22-33 (26.72 ± 1.95) x 2	12/12	360	0.12
	Mesobasic p-mastigophore	42-77 (58.61 ± 5.52) x 5-10 (7.02)	12/12	360	<0.001*

N total number of measured cnidocysts, *n* proportion of number of specimens in which each cnida was found. *Normality rejected. ▲ Analysis was not carried out due to the low N achieved

Table 5 Cnidom of *Bunodosoma zamponii*

Tissue	Cnida Type	Range (min-max) length (mean±sd) x width (mean) [µm]	n	N	p value
Tentacle	Basitrich	17-31 (23.73 ± 2.75) x 2-4 (2.75)	12/12	360	0.06
	Spirocyst	16-30 (22.63) x 2-4 (2.78)	12/12	360	<0.001*
Column	Basitrich I	12-28 (18.31 ± 1.92) x 2-4 (3.00)	12/12	360	<0.001*
	Basitrich II	11-19 (14.80 ± 1.40) x 2-3 (2.00)	12/12	360	0.17
	Spirocyst	17-33 (23.88 ± 3.53) x 2-4 (2.94)	12/12	121	0.08
	Holotrich I	28-56 (35.06 ± 5.90) x 4-6 (4.65)	12/12	329	<0.001*
	Holotrich II	17-29 (22.99 ± 2.47) x 3-6 (4.42)	11/12	330	0.009*
Mesenterial filament	Microbasic b-mastigophore I	30-58 (42.72 ± 4.12) x 4-8 (5.51)	12/12	360	0.04*
	Microbasic b-mastigophore II	18-30 (22.37 ± 2.20) x 3-5 (3.64)	12/12	360	0.02*
	Microbasic b-mastigophore III	8-23 (14.15 ± 2.40) x 2	12/12	360	0.02*
	Microbasic p-mastigophore	17-32 (22.71 ± 2.07) x 4-8 (5.34)	12/12	360	0.16
Actinopharynx	Basitrich	11-20 (15.10 ± 1.95) x 2-3 (2.16)	12/12	360	0.001*
	Spirocyst	16-33 (21.77 ± 2.68) x 2-3 (2.66)	10/12	118	0.60
	Microbasic b-mastigophore	20-33 (26.07 ± 2.56) x 2-4 (3.09)	12/12	360	0.08
	Microbasic p-mastigophore	17-26 (22.07 ± 2.10) x 3-7 (4.82)	11/12	69	▲
Acroraghi	Basitrich	10-25 (15.55 ± 2.77) x 2-3 (2.22)	12/12	360	<0.001*
	Spirocyst	17-42 (29.38 ± 4.71) x 2-4 (3.05)	12/12	360	0.008*
	Holotrich	33-63 (46.56 ± 5.85) x 4-6 (4.76)	12/12	360	0.75

N total number of measured cnidocysts, *n* proportion of the number of specimens in which each cnida was found. *Normality rejected. ▲ the analysis was not carried out due to the low N achieved

Table 7 Cnidom of *Aulactinia marplatensis*

Tissue	Cnida type	Range (min-max) length (mean±sd) x width (mean) [µm]	n	N	p value
Tentacle	Basitrich	16-26 (20.61 ± 0.52) x 2-4 (2.58)	12/12	360	0.08
	Spirocyst	16-26 (21.16 ± 2.04) x 2-4 (2.91)	12/12	360	0.19
Column	Basitrich I	12-23 (18.71 ± 1.63) x 2-3 (2.44)	12/12	360	<0.001*
	Basitrich II	7-15 (9.68 ± 1.54) x 2	12/12	360	<0.001*
	Holotrich	23-61 (34.50 ± 7.55) x 2-5 (3.38)	12/12	360	<0.001*
Mesenterial filament	Basitrich I	14-24 (18.36 ± 1.91) x 2-4 (2.38)	12/12	360	<0.001*
	Basitrich II	9-14 (11.45 ± 0.93) x 2-3 (2.01)	12/12	360	0.16
	Spirocyst	17-23 (20.36 ± 1.74) x 2-3 (2.91)	4/12	11	▲
	Microbasic b-mastigophore	23-40 (32.82±3.14) x 3-7 (4.76)	12/12	360	0.12
	Holotrich	31-57 (44.78 ± 6.93) x 3-4 (3.47)	5/12	19	▲
	Microbasic p-mastigophore	17-38 (22.82 ± 3.57) x 4-9 (5.55)	12/12	360	<0.001*
Actinopharynx	Basitrich I	10-24 (17.84 ± 2.17) x 2-5 (2.34)	12/12	360	0.09
	Basitrich II	21-34 (26.34 ± 2.02) x 3-5 (3.51)	12/12	360	0.35
	Basitrich III	7-14 (10.85 ± 1.16) x 2-3 (2.01)	12/12	360	<0.001*
	Spirocyst	14-34 (20.29 ± 3.37) x 2-4 (2.83)	12/12	315	0.21
	Microbasic amastigophore	16-33 (23.40 ± 3.35) x 4-9 (5.56)	12/12	360	0.002*

N total number of measured cnidocysts, *n* proportion of the number of specimens in which each cnida was found. *Normality rejected. ▲ Analysis was not carried out due to the low N achieved

Table 9 Cnidom of *Bunodactis octoradiata*

Tissue	Cnida type	Range (min-max) length (mean±sd) x width (mean) [µm]	n	N	p value
Tentacle	Spirocyst	12-35 (23.33 ± 4.25) x 2-5 (3.10)	10/10	300	0.341
	Basitrich I	11-35 (21.23 ± 4.23) x 2-5 (2.96)	10/10	300	<0.001*
	Basitrich II	12-52 (26.61 ± 5.73) x 2-6 (3.98)	10/10	300	<0.001*
Column	Basitrich I	10-27 (16.27 ± 2.97) x 2-4 (3.01)	10/10	300	<0.001*
	Basitrich II	10-35 (19.86 ± 5.76) x 2-6 (3.48)	10/10	300	<0.001*
Mesenterial filament	Basitrich I	10-35 (22.99 ± 4.86) x 2-5 (3.17)	10/10	300	0.019*
	Basitrich II	10-37 (25.38 ± 5.22) x 3-5 (3.67)	10/10	300	0.007*
	Microbasic p-mastigophore	10-34 (23.26 ± 4.26) x 3-5 (3.32)	10/10	300	0.113
Actinopharynx	Basitrich	19-48 (31.26 ± 4.72) x 3-6 (4.25)	10/10	300	<0.001*
	Microbasic p-mastigophore	32-15 (22.96 ± 2.88) x 3-5 (3.55)	10/10	300	<0.001*

N total number of measured cnidocysts, *n* proportion of the number of specimens in which each cnida was found. *Normality rejected

Figure 1

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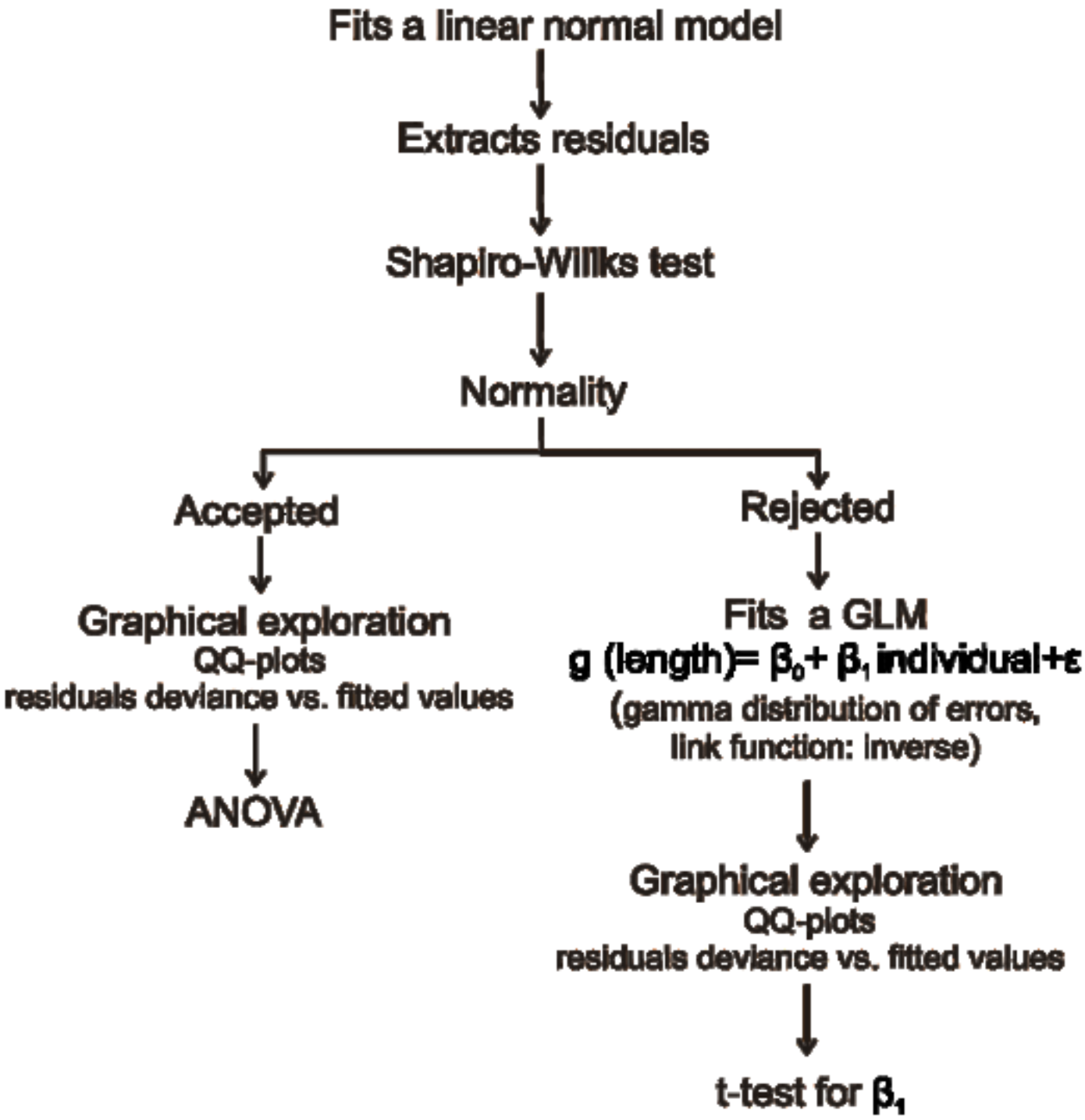

Figures - Electronic Supplementary Material