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# "Nose-metrics" of wild southern elephant seal (*Mirounga leonina*) males using image analysis and geometric morphometrics

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## Abstract

The elephant seal (genus *Mirounga*) proboscis is a textbook example of an exaggerated secondary sexual trait, whose function is debated. The proboscis can be related to sexual status advertising, emission of aggressive vocalizations, and/or female mating choice. The study of the proboscis is complicated, because it is a soft trait that needs to be studied when males vocalize and it is thus expanded. Here, we combined field stimulation experiments, 2D photogrammetry, and geometric morphometrics, to study the proboscis of wild elephant seals in natural conditions. The goal of our study was twofold: (a) to demonstrate that photogrammetry and geometric morphometrics can be effectively applied in the field to wild, large, non-sedated mammals; (b) to study the proboscis shape development during maturation. We found that it is possible to accurately estimate the proboscis size and shape using photographs of vocalizing males taken in the field. Moreover, we showed that mature and non-mature males differ not only in proboscis size but also in its shape, a difference that is largely allometric and can have important effects on the frequency structure and individual signature of male vocalizations. These results open new avenues for future research on this enigmatic structure, its function in aggressive displays and potential role in sexual selection, and also exemplify a very promising approach, that could be applied in field studies of other large mammals.

**KEYWORDS**
2D photogrammetry, measurement error, pinnipeds, proboscis, semilandmarks

## 1 | INTRODUCTION

The elephant seal (genus *Mirounga*) proboscis has been a textbook example of an exaggerated secondary sexual trait from the very beginning of evolutionary biology (Darwin, 1871). In both species of elephant seal (the southern, *M. leonina* (Linneaus, 1758), and the northern, *M. angustirostris* Gill, 1866) males develop an elongation of the nostrils that results in a true proboscis in breeding males. The sexual dimorphism in the nostrils becomes evident at age three to four (Laws, 1953), but the proboscis shows a significant enlargement and shape change during the post-pubertal growth spurt showed by males between sexual maturity and adulthood (McLaren, 1993). Although the basic anatomy and development of the elephant seal proboscis are well known (Laws, 1953), a demonstration of its function(s) remains elusive.

We previously showed that both body size and age have an independent effect on the proboscis size that is apparently under a positive directional phenotypic selection (Sanvito, Galimberti, & Miller, 2007a). In fact, the proboscis development is a rather accurate morphological cue of male age, permitting the assessment

of age class with one-year accuracy (unpublished data). Elephant seals have a polygynous mating system based on harem defense, harem holders are the most dominant males, and dominance is established through dyadic agonistic interactions (Sandegren, 1976; McCann, 1981). The proboscis is an important component of the visual displays of male interactions (Sanvito, Galimberti, & Miller, 2007b) that determine a large part of the variance in reproductive success (Le Boeuf and Reiter 1988). During the breeding season, males keep the proboscis fully expanded most of the time, while during the molt, the only other time of the year in which they are on land, they keep it contracted (Laws, 1953). Therefore, the expansion of the proboscis can be involved in the advertisement of breeding status (Laws, 1953; Sandegren, 1976) and can be related to the testosterone level of the male (unpublished data). The proboscis is connected to the main vocal tract of the male and, therefore, can have a role in the emission of aggressive vocalizations that are probably the most important component of male agonistic behavior (Sanvito et al., 2007b). We previously showed that males emit vocalization with a frequency structure that cannot be fully explained by the buccal part of the vocal tract, and we suggested that the lowest format frequency observed in spectrograms of male vocalizations can be due to its nasal part (Sanvito et al., 2007b). The proboscis can also be involved in the female choice of mating partners, although the elephant seal harem mating system, and the effectiveness of male control of female movements (Galimberti, Boitani, & Marzetti, 2000), greatly constrain the scope of female choice in these species (but see Cox & Le Boeuf, 1977).

The starting point to solve the elephant seal proboscis puzzle should be the gathering of accurate data about its size, shape, and development. The main problem in the study of the proboscis is that it is a soft tissue that should be studied when fully expanded to generate meaningful data (Sanvito et al., 2007a). This means that one has to measure the proboscis on live, wild, elephant seal males, during their natural behavior, and, in particular, when they vocalize. In principle, the study of morphology of wild animals can be carried out using photogrammetry, that is, by taking standardized photographs in which a metric scale is included in the photograph frame, such as, for instance, by using parallel lasers (Bergeron, 2007). Photogrammetry is a non-invasive method that is now frequently used in field studies because it permits to estimate body size of wild animals without capturing and/or sedating them (Berger, 2012), but the use of photogrammetry to study morphology of wild animals is definitely less frequent (Breuer, Robbins, & Boesch, 2007), and its application to soft tissues in naturally behaving individuals is even rarer (Sonnweber et al., 2013). Indeed, most morphometric measures of wild animals are obtained when the subjects are captured, hand restrained, or chemically sedated. However, this approach cannot be applied to many secondary sexual traits, including the elephant seal proboscis, that are under the subject control, so that their size and shape depends on individual behavior and motivation (Frey, Gebler, Fritsch, Nygrén, & Weissengruber, 2007; Frey, Volodin, & Volodina, 2007).

Thus, in order to explore whether we can obtain accurate data on this soft-tissue structure using photogrammetry in the field, we elicited vocalizations in immature and fully mature males and took photographs of the proboscis in side view. In total, at different times, we made three photographs of each individual in an almost perfectly balanced sample of 20 immature and 23 mature males, and we digitized a set of anatomical landmarks twice on each image, producing a total of 258 observations. On these 2D images, we applied Procrustean geometric morphometrics (Adams, Rohlf, & Slice, 2004; Cardini, 2013) to assess:

1. Whether the measurement error of size and shape was low, and the repeatability high, despite the difficulties to obtain standardized pictures of the fully expanded proboscis in naturally behaving seals.
2. Whether proboscis size and shape differ in means and variance between the mature and immature males.
3. Whether growth, developmental, and allometric trajectories can be estimated to explore the ontogenetic patterns of morphological change in such an extreme sexually dimorphic structure.

## 2 | MATERIALS AND METHODS

### 2.1 | Field work and 2D photogrammetry

We studied southern elephant seals at Sea Lion Island, the main breeding colony of the species in the Falkland Islands, that shelters about 620 breeding females and about 60 breeding males. We marked all breeding males of the population by numbered plastic tags at birth, so their age was known (6–12 years). We classified males as immature (age 6–9) and mature (age ≥ 10). We also marked males by painting names on their flanks to permit identification from the distance. Details on the general field protocol can be found in Galimberti and Boitani (1999). We estimated body size using a previously validated 2D photogrammetric method (Galimberti, Sanvito, Braschi, & Boitani, 2007).

To obtain photographs of the male proboscis, we elicited a behavioral reaction by stimulation. Briefly, an operator approached the elephant seal male from the front and that elicited a behavioral sequence of vocalization and aggression analogous to the natural reaction to the approach of another male. A similar direct stimulation approach was shown to be effective in previous studies of elephant seal vocalizations (Sanvito & Galimberti, 2000a,b; Sanvito et al., 2007a; Sanvito, Galimberti, & Miller, 2008) and proboscis morphology (Sanvito et al., 2007b). The comparison of behavioral sequences and acoustic features of vocalizations showed that they were (within each male) almost equal between natural (toward other seals) and stimulated (toward operator) events (Sanvito & Galimberti, 2000a,b). In these studies, no habituation effect was observed, and males reacted with their normal display even when regularly stimulated once per week for the whole length of breeding season (10–14 weeks). Thus, while the male was vocalizing, with the proboscis expanded, the operator kept a surveying pole in front of the subject, aligned to

its mid axis using the lower canine teeth as reference. At the same time, another operator took high resolution photographs of the proboscis using a digital camera (E-M10 Mark III, Olympus) fitted with a zoom lens (14–42 mm), keeping the camera plane parallel to the sagittal plane of the subject, and including at least 20 cm of the surveying pole in the photograph frame. Using this protocol, as in our previous study (Sanvito et al., 2007a), we took side photographs in order to capture the midplane outline of the proboscis. Overall, we photographed 43 breeding males (20 immature and 23 mature).

All field work procedures were carried out in accordance with best practice guidelines for the study of wild mammals (Gales et al., 2009; Sikes & Gannon, 2011).

## 2.2 | Photographs and landmark digitization

We carried out a preliminary screening of photographs to select, for each male, the photographs with the most expanded proboscis. For each male, we selected three independent photographs, that is, three photographs taken in different days and/or during different behavioral sequences. We digitized landmarks on the proboscis outline using tpsDig software (version 2.30, Rohlf, 2015), repeating the digitization two times on each photograph. Landmarks (4) and semilandmarks (19) are shown in Figure 1, which also shows the total sample superimposed after sliding the semilandmarks (see below). As in Sanvito et al. (2007a), we defined the proximal region of the proboscis between landmarks 3 and 4 as the "first bump" and the more rostral one, between landmarks 1 and 3, as the "second bump" (Figure 1).

We used two landmark configurations in the analyses, referred to as SL (full set of 23 points including slid semilandmarks, all points of Figure 1) and OL (the reduced configuration of four ordinary "fixed" landmarks, number 1–4 in Figure 1). We performed all analyses on both configurations, because semilandmarks can greatly help to quantify form in structures with few clearly corresponding landmarks (Gunz & Mitteroecker, 2013), but these

"special" points share some of the limitations of traditional methods for the analysis of outlines (e.g., EFA—O'Higgins, 1997; Oxnard & O'Higgins, 2011), and largely increase the number of variables in the analysis, which can lead to unreliable findings due to an unfavorable ratio between data dimensionality ($p$) and sample size ($N$; Bookstein, 2017).

## 2.3 | Geometric morphometrics

We performed all analyses on size and shape data obtained using Procrustes superimposed landmarks (Rohlf & Slice, 1990; Adams et al., 2004; Cardini, 2013). We estimated the size of the proboscis outline by the centroid size (CS = the square root of the sum of squared distances between each point and the centroid of the point configuration), that measures the dispersion of the landmarks around the center of the configuration. We obtained shape coordinates by dividing the raw landmark coordinates by CS, centering all specimens on the centroid, and minimizing rotational differences. For the full configuration (SL), we slid semilandmarks along the outline of the proboscis so that the total sum of squared Procrustes distances to the sample mean was minimized (minPRD approach; Bookstein, 1997). Sliding semilandmarks is an expedient to improve the mathematical correspondence of points that have no clear one to one correspondence, unlike conventional landmarks. We tested the sensitivity to the choice of the sliding approach by calculating the correlations between shapes (i.e., the matrices of pairwise Procrustes shape distances) and CS estimated from Procrustes superimposed unslid data (i.e., with all points treated as if they were standard landmarks) or data with semilandmarks slid either using minPRD or the main alternative method, which minimizes the bending energy using the thin plate spline smoothing function (minBE; Bookstein, 1997). We performed these correlational analyses in NTSYSpc (version 2.21r; Rohlf 2009).

We illustrated patterns of shape variation (i.e., maturity class differences and ontogenetic trajectories) using the SL configuration and



**FIGURE 1** Landmark configurations: (a) landmarks and semilandmarks on a male photograph taken during the vocalization; (b) landmarks (indicated by arrows), and semilandmarks with a wireframe (a set of lines connecting the points along the outline); (c) superimposed slid full configuration. In (a) and (b), and all other shape diagrams in the next figures, the first and last landmarks are emphasized using different symbols to aid the reader to correctly interpret the orientation of the nose (which is, however, always the same in all figures, with the tip of the nose to the right)
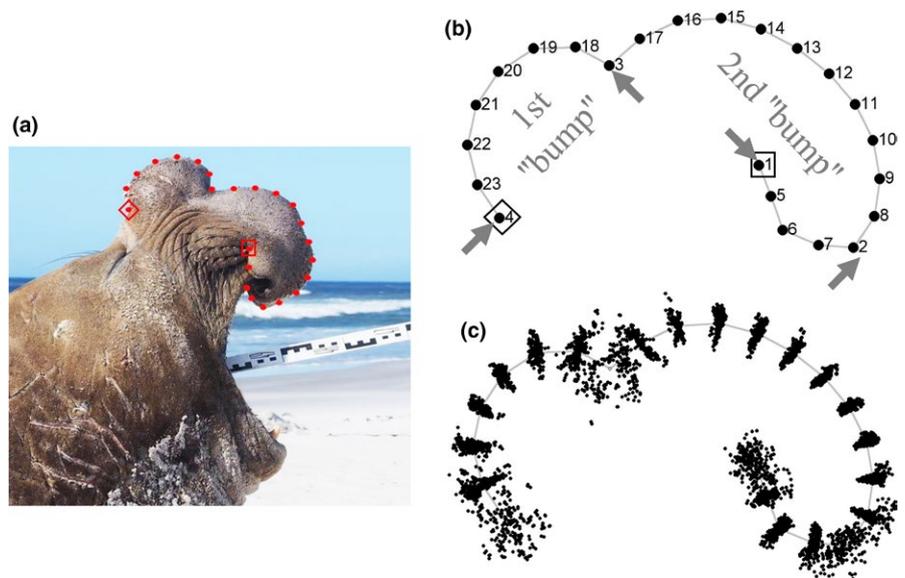
wireframe diagrams (Klingenberg, 2013). We obtained wireframes by linking pairs of landmarks/semilandmarks with lines (Figure 1b), that can be optionally allowed to bend ("soft wireframes") using the thin plate spline smoothing function.

We superimposed SL data in TPSRelw (version 1.69; Rohlf, 2015) by sliding semilandmarks using the maximum number of possible iterations (40), to guarantee an optimal alignment. We performed all further analyses in MorphoJ (version 1.06d; Klingenberg, 2011). We also analyzed the SL slid-superimposed data in TPSSmall (version 1.32; Rohlf, 2015) to test the approximation of the curved Procrustes shape space to its projection on the flat Euclidean space, in which statistical analyses are performed.

## 2.4 | Statistical analyses

We assessed measurement error in MorphoJ using a Procrustes ANOVA for both size and shape (Klingenberg, Barluenga, & Meyer, 2002; Viscosi & Cardini, 2011; Fruciano, 2016), in which maturity was used as the fixed factor, individual identity as the random factor, and replicated photographs and digitizations as the error terms.

Each level of the ANOVA was nested in the previous one. Although in this, and in following analyses, $p$ values were estimated, the main focus was on the relative magnitude of the different effects, measured by the percentage of sum of squares accounted for by each factor ($R^2$) and the repeatability. Repeatability was calculated both for univariate and multivariate data following Fruciano (2016).

We used a phenogram of Procrustes shape distances to assess the number of replicas of each individual (three photographs × two digitizations = 6 replicas) that actually clustered together "within" the individual (see figure 5 of Daboul, Ivanovska, Bülow, Biffar, & Cardini, 2018, for an example). We computed an unweighted pair group method using arithmetic average (UPGMA) phenogram in PAST (version 2.17c; Hammer, Harper, & Ryan, 2001). That number may range from zero individual replicas clustered together, that implies minimum repeatability, to all six replicas of an individual clustering together, which implies the maximum repeatability for that individual. If measurement error is negligible, differences between replicas should be small relative to inter-individual differences, and therefore, replicas can be averaged "within" each individual, and individual averages of size and shape used for all further analyses, as described below.

We tested the differences in mean size and shape between the two maturity classes in MorphoJ using a regression onto a dummy variable (0 for immature and 1 for mature males) and a permutation test (10,000 replicates) to estimate significance. We assessed how accurately age groups could be predicted based on size or shape using a leave-one-out cross-validated linear discriminant analysis (DA). Finally, we tested differences in the magnitude of variation within each maturity class using a permutational version of Levene's test (Hallgrímsson et al., 2006; Nagorsen & Cardini, 2009), carried out in PAST after computing within group absolute deviations from the group mean for both size and shape. For shape, the deviation

was the Procrustes distance of each individual to the mean of its group, computed in NTSYSpc. To perform the test, we regressed deviations onto a dummy variable for the groups and assessed significance using a permutation test (10,000 replicates).

The last set of analyses explored ontogenetic patterns using averaged data within each year of age from six (the youngest group of immature males) to 12 (the oldest adults). We performed all analyses using regressions (with permutation test, 10,000 replicates) of size onto age (growth), shape onto age (development), and shape onto size (allometry). For shape, regression was multivariate (i.e., all shape variables used as dependent variables) and we used the vectors of coefficients to compute the angle between different trajectories in MorphoJ (e.g., developmental vs allometric trajectory).

## 3 | RESULTS

In the following subsections of the Results, we briefly anticipate a few points of the Discussion. These are comments on very specific methodological issues that would be a distraction in the Discussion and are better contextualized if mentioned next to the corresponding results.

## 3.1 | Preliminary analyses

The approximation of the curved shape space to its projection on a flat Euclidean space was found to be excellent (range of pairwise Procrustes shape distances: SL, 0.0088–0.2581; OL, 0.0294–0.2661) with very high correlations (≥0.99) of distances in the two shape spaces and slopes of regressions of Procrustes onto Euclidean distances being equal to 1.00 in both SL/OL.

The analysis of the sensitivity of size and shape data (including all replicas) in relation to the mathematical treatment of semilandmarks showed that sliding introduced negligible differences. The correlations between matrices of pairwise Euclidean shape distances were all very high (unslid vs minBE slid, unslid vs minPRD slid, and minBE vs minPRD slid, all being equal to $r = 0.96$). For centroid size (CS), corresponding correlations after rescaling slid data in TPSRelw were also very high (>0.99). Therefore, the choice of sliding approach had a negligible effect on the results.

## 3.2 | Congruence of reduced and full configurations

The analyses of both configurations produced fully congruent results, despite the four landmark configuration contained less than 20% of the putative morphological information available in the full set of 23 points on the proboscis outline. For instance, $R^2$s in the ANOVAs of size and shape using either OL or SL had a correlation ≥0.99 between analyses using one or the other set of points. Similarly, $R^2$s for maturity class differences in both size and shape were almost identical. Indeed, the correlation of CS estimated from the two configurations was 0.96 and that between the corresponding matrices of Procrustes shape distances (computed pairwise

**TABLE 1** Procrustes ANOVA of centroid size (CS - white background) and shape (light gray background) for both configurations, with age class as main factor, individual as random factor, and photograph/digitization as error terms; the ratio (IE) of individual $R^2$ to total measurement error (sum of photograph and digitization $R^2$) is shown, as well as the estimate of repeatability

| | | Effect | SS | MS | df | F | P(param.) | Pillai_tr. | P(param.) | $R^2$ (%) | IE ratio | Repeatability |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| CS | SL | Age class | 11,596 | 11,595.76 | 1 | 28.8 | <0.0001 | — | — | 39.7 | — | — |
| | | Individual | 16,538 | 403.36 | 41 | 32.3 | <0.0001 | — | — | 56.6 | 15 | 0.93 |
| | | Photograph | 1,073 | 12.48 | 86 | 57.5 | <0.0001 | — | — | 3.7 | — | — |
| | | Digitization | 28 | 0.22 | 129 | — | — | — | — | 0.1 | — | — |
| | | Total | 29,235 | — | — | — | — | — | — | 100.0 | — | — |
| | OL | Age class | 919 | 919.27 | 1 | 23.1 | <0.0001 | — | — | 34.7 | — | — |
| | | Individual | 1,631 | 39.79 | 41 | 38.8 | <0.0001 | — | — | 61.6 | 17 | 0.94 |
| | | Photograph | 88 | 1.03 | 86 | 15.6 | <0.0001 | — | — | 3.3 | — | — |
| | | Digitization | 8 | 0.07 | 129 | — | — | — | — | 0.3 | — | — |
| | | Total | 2,647 | — | — | — | — | — | — | 100.0 | — | — |
| Shape | SL | Age class | 0.145 | 0.00346 | 42 | 4.0 | <0.0001 | — | — | 7.7 | — | — |
| | | Individual | 1.50 | 0.00 | 1,722 | 16.7 | <0.0001 | 25.28 | <0.0001 | 80.1 | 7 | 0.85 |
| | | Photograph | 0.189 | 0.00005 | 3,612 | 7.4 | <0.0001 | 24.8 | <0.0001 | 10.1 | — | — |
| | | Digitization | 0.039 | 0.00001 | 5,418 | — | — | — | — | 2.1 | — | — |
| | | Total | 1.878 | — | — | — | — | — | — | 100.0 | — | — |
| | OL | Age class | 0.485 | 0.12131 | 4 | 4.6 | 00.0015 | 0.3 | 0.0179 | 9.2 | — | — |
| | | Individual | 4.30 | 0.03 | 164 | 20.9 | <0.0001 | 3.39 | <0.0001 | 81.3 | 9 | 0.88 |
| | | Photograph | 0.432 | 0.00125 | 344 | 9.2 | <0.0001 | 3.3 | <0.0001 | 8.2 | — | — |
| | | Digitization | 0.070 | 0.00014 | 516 | — | — | — | — | 1.3 | — | — |
| | | Total | 5.288 | — | — | — | — | — | — | 100.0 | — | — |

between all specimens) was 0.92. For shape, the correlation was virtually unchanged (0.92) even if only the first four PCs (88% of total shape variance) of the full configuration were used. Therefore, the main pattern of variation was largely captured by using just the four landmarks.

Overall, estimates of size and proportions based on four landmarks produced an accurate numerical description of the main features of the elephant seal proboscis. Using just four landmarks (i.e., once the loss of degrees of freedom in the superimposition is considered, only four variables) avoids an inflation in the number of variables (*p*), and thus a potentially unfavorable *p/N* ratios (Bookstein, 2017), while preserving accuracy. Nevertheless, one might still want to explore the full configuration for a more effective visualization of shape differences.

## 3.3 | Measurement error

The main results of the assessment of measurement error are presented in Tables 1 and 2. The component of the error due to differences among photographs taken at different times was five to 40 times larger than the digitizing error (Table 1). This photographic error is probably the sum of the true error that reflects the difficulties to standardize the pictures taken in the field, and the variation in breeding status of the male (that changes along the season), its

short-term motivation, and the intensity of its reaction to the operator approach. However, despite this various sources of spurious variability, repeatability was high (0.85–0.94) and individual variation, regardless of maturity class, was seven to 17 times larger than total measurement error, the latter accounting between 4% and 12% of total variance in, respectively, size and shape. For shape, the phenogram consistently showed high repeatability for most specimens, with 74% (OL) to 93% (SL) of individuals having at least four out of six replicas clustering together (Table 2).

## 3.4 | Differences between maturity classes

Immature and fully mature males showed highly significant differences in the morphology of the proboscis (Table 3; Figures 2–3), larger for size (ca. 36%–41% of total size variance) than for shape (9%–10% of variance). However, classification accuracy in the cross-validated DAs varied from low (ca. 60%) to moderate (ca. 75%), with size always performing better than shape, and with SL shape providing the lowest accuracy. This latter observation, together with the already mentioned congruence between OL and SL shape distances, suggest that semilandmarks might be making the results "noisier," although they allow a better visualization. In fact, wireframe diagrams for means (Figure 3) indicate that mature individuals not only have bigger proboscis but also show a relatively broader expansion

of the second bump, that becomes larger, deeper, and more concave (Figure 3).

None of the comparison of the magnitude of variance in size and shape between the maturity classes was significant (Table 4). However, in all instances, except OL size, immatures showed larger variance than mature males. Immature males, that are still growing, likely vary more in proboscis size and shape than fully adult males, that have already reached their maximum growth, and the lack of significance might be due to the small sample size and low power of tests. Nevertheless, estimates of variance are known to be strongly affected by sampling error (Cardini, Seetah, & Barker, 2015, and references therein) and require very large samples for effective testing of differences.

### 3.5 | Exploration of ontogenetic patterns

All components of ontogenetic change were statistically significant and had very large $R^2$s, accounting for 50%–80% of variance in size or shape (Table 5). Thus, not only on average the proboscis kept growing, and was generally larger in older individuals, but also most of the variation in shape was allometric, with allometry and development having almost collinear trajectories, as indicated by the small angle between their vectors (<13° in the full multivariate shape space). The pattern of allometric change was very similar to that seen for the differences between immature and mature males, as suggested by the visualization of shape changes (compare Figures 3 and 4), and by the small/moderate (15–27° for, respectively, SL and OL) angles formed by the corresponding vectors.

Thus, as expected, if age class were tested again after regressing out allometric shape (results not shown), they would explain a tiny amount (<1%) of shape variance regardless of dataset, and neither SL nor OL would be any longer significant. However, the test using "size-corrected data" (Klingenberg, 2011, and references therein) was not included in the main analysis, and it is only briefly mentioned here, because it requires large samples to accurately estimate allometries within age classes and assess whether they are parallel or not (a fundamental assumption for "size-correcting" the data using a MANCOVA model). Using the available data, it seems that in fact this

**TABLE 2** Proportions (as counts out of total $N = 43$ and as percentages) of specimens with all six, just five, four etc., replicas clustering together within individual in UPGMA phenograms of OL and SL shape

|  | SL | | OL | |
|---|---|---|---|---|
|  | Counts | % | Counts | % |
| All 6 | 25 | 58 | 15 | 35 |
| 5 | 1 | 2 | 5 | 12 |
| 4 | 14 | 33 | 12 | 28 |
| 3 | 1 | 2 | 5 | 12 |
| 2 | 2 | 5 | 6 | 14 |
| None | 0 | 0 | 0 | 0 |

assumption may not be met and immature and mature allometries might follow different trajectories with observed angles of 42–52° for, respectively, SL and OL datasets.

## 4 | DISCUSSION

### 4.1 | Photogrammetry, sampling, and morphometrics in wild animals

The study of morphometrics is largely focused on hard-tissue structures, and the application to soft tissues is much more limited, with the notable exception of humans. This is mostly due to the difficulty to obtain standard images and digitize landmarks with high repeatability on soft tissues (Daboul et al., 2018). These difficulties are clearly amplified in studies of wild subjects displaying their normal behavior in natural conditions (Sonnweber et al., 2013). Indeed, previous applications to soft tissues of live non-human animals, such as the one by Alarcón-Ríos, Velo-Antón, and Kaliontzopoulou (2017), were typically using captured and/or sedated individuals.

The need to work with high ethical research standards to preserve the welfare of study animals and do not interfere with the elephant seal natural behavior (Wade, Zalucki, & Franzmann, 2005), as well as the Falkland Islands very strict regulations for

**TABLE 3** Differences in size (white background) and shape (light gray background) of mature and immature males: permutation test of mean differences and classification accuracy (hit-rate) in leave-one-out DAs (using the first eight PCs explaining 95.2% of variance for SL shape and all PCs, i.e., 100% of variance, for OL)

|  | SL | | | | | OL | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
|  | Diff. in means | | Hit-rate of jacknifed DA | | | Diff. in means | | Hit-rate of jacknifed DA | | |
|  | $p$ | $R^2$ | | Mat. | Imm. | Tot. | $p$ | $R^2$ | | Mat. | Imm. | Tot. |
| CS | <0.0001 | 41.2% | Mat. | 83% | — | — | <0.0001 | 36.0% | Mat. | 74% | — | — |
|  | — | — | Imm. | — | 70% | — | — | — | Imm. | — | 75% | — |
|  | — | — | Tot. | — | — | 77% | — | — | Tot. | — | — | 74% |
| Shape | 0.008 | 8.8% | Mat. | 57% | — | — | 0.007 | 10.1% | Mat. | 70% | — | — |
|  | — | — | Imm. | — | 65% | — | — | — | Imm. | — | 65% | — |
|  | — | — | Tot. | — | — | 60% | — | — | Tot. | — | — | 67% |
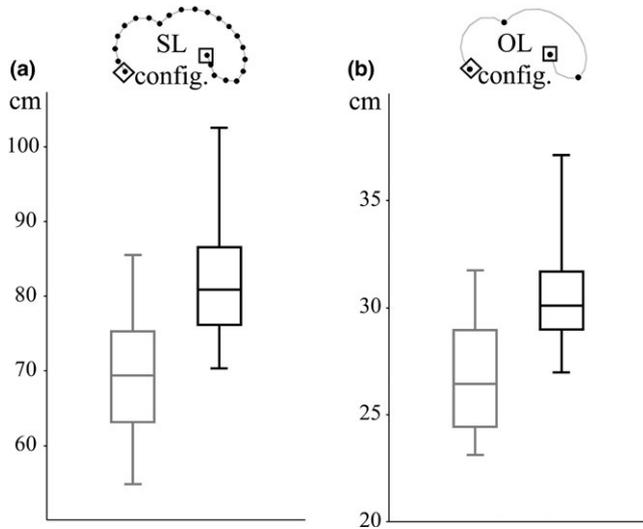
**FIGURE 2** Boxplots of proboscis centroid size, comparing immature (gray) and mature (black): (a) full configuration (SL); (b) reduced configuration using only landmarks (OL)

measurement of naturally behaving animals, precision and accuracy may be reduced (Harris, 2012).

The elephant seal proboscis is a soft tissue that changes in size and shape in relation to the male motivation and status. Thus, to obtain standardized images of the fully inflated proboscis one has to take this source of variability into account by "controlling" for the type of behavioral response. To this aim, we elicited aggressive behavior that accurately simulates the male elephant seal natural agonistic behavior. As anticipated in the Methods, we previously demonstrated that the human-induced behavioral sequence is similar to the one shown during natural interactions among males and that the corresponding vocalizations have the same time and frequency structure of natural aggressive vocalizations (Sanvito & Galimberti, 2000a,b). However, even if the protocol for image acquisition is standardized and had already been used and published, compared to traditional morphometrics using photographs of elephant seals (Galimberti et al., 2007; Sanvito et al., 2007a), geometric morphometrics is more complex. There might be difficulties to obtain reliable landmarks on the proboscis and also measurement error is generally more pronounced in shape data (e.g., Viscosi & Cardini, 2011, and Cardini, 2014) than in traditional measurements of size. Thus, it was crucial to accurately assess the main sources of error, that could artificially inflate variation, reduce statistical power, and introduce biases (Arnqvist & Maartensson, 1998; Fruciano, 2016; Daboul et al., 2018).

wildlife protection, make low-invasive approaches to data collection fundamental (Pauli, Whiteman, Riley, & Middleton, 2010). Photogrammetry provides an effective tool to non-invasively measure phenotypic variation in the field (Berger, 2012; Miller, Best, Perryman, Baumgartner, & Moore, 2012; Breuer et al., 2007; Bergeron, 2007). However, as other methods for indirect

**TABLE 4** Differences in size (white background) and shape (light gray background) of mature and immature males: Levene's test for the magnitude of variance

| | SL | | | OL | | |
|---|---|---|---|---|---|---|
| | Levene | Variance | | Levene | Variance | |
| | p | Mat. | Imm. | p | Mat. | Imm. |
| CS | 0.4444 | 61.1 | 74.3 | 0.7470 | 6.7 | 6.6 |
| Shape | 0.0855 | 0.0051 | 0.0073 | 0.2552 | 0.0156 | 0.0197 |
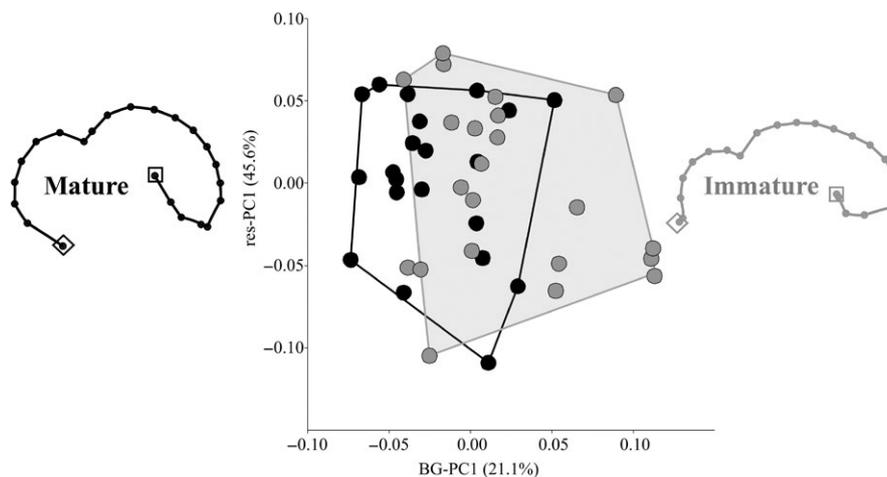


**FIGURE 3** Scatterplot of between group PC1 (BG-PC1) and residual non-between group PC1 (res-PC1) of SL shape (percentages of total variance accounted for by each axis are in parentheses). Immature males are shown in gray and mature ones in black, with convex hulls emphasizing the two groups. Mean shapes of the two age classes are plotted at the opposite extremes of BG-PC1, with differences magnified five times

**TABLE 5** Exploratory ontogenetic regressions using averaged data within years of age (6, 7, 8 etc. up to 12 years old). The angles between multivariate allometric and developmental vectors are for SL/OL, respectively, 12.5°/9.9°)

|  | SL | | OL | |
|---|---|---|---|---|
|  | *p* | $R^2$ (%) | *p* | $R^2$ (%) |
| Growth | 0.0021 | 82.7 | 0.0062 | 79.5 |
| Allometry | 0.0062 | 66.5 | 0.0044 | 69.3 |
| Development | 0.0275 | 49.3 | 0.0270 | 56.3 |

The number of subjects analyzed was relatively small, but not unusual in geometric morphometrics (Cardini & Elton, 2007; Cardini et al., 2015). Besides, the assessment of measurement error was based on a fairly large number of replicas (three separate photographs per male, each digitized twice, with 258 observations in total). Also, the sample of this study was somehow unusual in that data were collected during a single season in a single local population of elephant seals. In this respect, as well as increasing samples and exploring more specific morpho-functional hypotheses, it will be interesting to expand the study to other populations of the same species, as well as to their closest relative, the northern elephant seal. Although in the northern elephant seal females have somehow enlarged nostril compared to southern species females, only males of both species develop a true proboscis, so the trait is strongly dimorphic. Interestingly, despite the larger size of southern elephant seal males, the development of the proboscis is more extreme in the northern species (Le Boeuf & Laws, 1994) but interspecific comparisons of their shape are lacking.

Our study showed that measurement error, although relatively large if compared to hard tissues, was much smaller than variation among individuals and age classes. Most of the error was due to differences between photographs of the same male. This error is probably a combination of a true error, due to the difficulties to standardize pictures of live animals in the field, and of the natural variability in the degree of proboscis expansion during vocalizations. This second component of measurement error depends on testosterone levels, breeding status, and small differences in motivational status (Laws, 1956; Sanvito et al., 2007a).

Despite all the difficulties, having obtained such a relatively small amount of measurement error seems more than promising for future studies. In fact, not only the error was small but results were also robust to the choice of the landmark configuration (with semilandmarks or reduced to four landmarks only). This high congruence between SL and OL is particularly important, as it suggests that SL data, despite an unfavorable *p/N* ratio (Bookstein, 2017), were accurate. Most of the morphometric signal was, however, accurately captured by the four landmarks, whereas the semilandmarks, although helpful in the visualization, introduced an amount of noise in the dataset. Indeed, not only DAs classification accuracy was smaller using SL, but also repeatability was slightly smaller than using OL, despite the larger number of replicas clustering "within individual" in the phenogram of SL shape. Nevertheless, repeatability was generally high and measurement error negligible in all proboscis datasets, which is fundamental to open new avenues for future research on this enigmatic structure, its function in aggressive displays and potential role in sexual selection. Indeed, the elephant seal proboscis was one of the examples mentioned by Darwin (1871) to describe the theory of sexual selection and is a textbook case of secondary sexual trait (Andersson, 1994).

## 4.2 | Ontogeny and function

We used the tests of differences between age groups and the exploratory analyses of ontogenetic patterns as examples of applications of our approach to biological questions. Results, although very preliminary, are interesting, with large effect sizes and robust patterns.

Young males not only have smaller proboscis, but also a remarkably different shape that over the years develops from a fairly
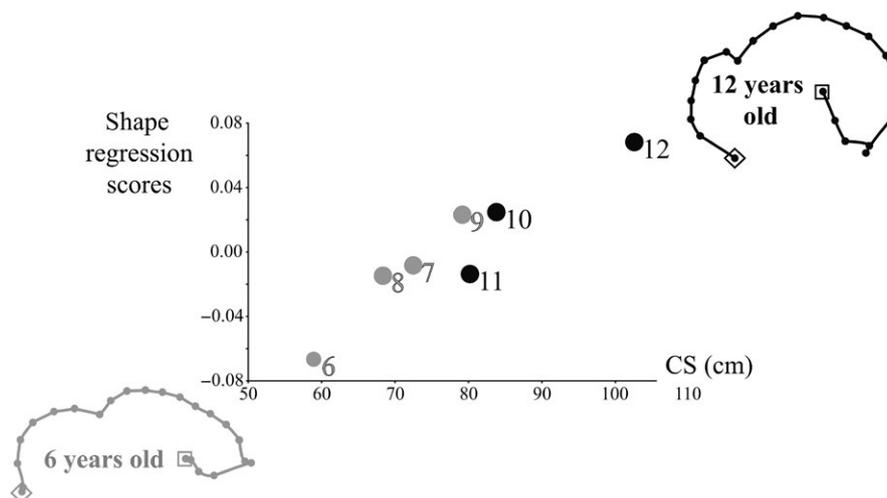


**FIGURE 4** Allometric trajectory for the SL averages of immature (6–9 years old, gray) and mature (10–12 years old, black) males. Opposite extremes of the allometric trajectory, magnified two times, are shown at the sides of the scatterplot

straight tubular shape, split by a main fold in two halves of approximately similar size, to a much more curved structure, with a highly enlarged second bump. This fundamental change in the proboscis shape during aging may be related to the age-specific changes in the frequency structure of male vocalizations (Sanvito et al., 2008).

Our preliminary analysis also suggests that a large component of shape change in the proboscis is allometric with likely differences between age groups. It will be interesting in the future to include younger individuals to better understand the way a most dimorphic feature, such as the elephant seal proboscis, develops. Although the sexual dimorphism of the nostrils is evident also in very young seals, the real development of the proboscis begins after puberty, when elephant seal males experience an incredible acceleration of the growth rate that produces the largest growth spurt of all mammals (McLaren, 1993). Despite a very large individual variation (McCann, 1981), during the post-pubertal growth, and until full adulthood, the proboscis increases in size and becomes gradually more prominent (Laws, 1953; Sanvito et al., 2007a).

The function of elephant seal proboscis remains elusive and largely speculative (Laws, 1956; Sandegren, 1976; McCann, 1981). The proboscis can have a role in the visual displays (Sandegren, 1976) and in the emission of vocalizations (Laws, 1956) that males use to establish dominance; it can be related to the hormonal status of the male, and testosterone in particular (Griffiths, 1984); and it is a potential cue for female choice (Cox & Le Boeuf, 1977). However, for now, there is strong support only for a role of the proboscis in the vocalizations. In accordance with the source–filter theory of sound production in mammals (Taylor & Reby, 2010), the vocal tract acts as a filter for frequencies produced by the vocal folds, providing an anatomical intermediary for the honesty of signaling in elephant seals (Sanvito et al., 2007b) and other mammals (Fitch & Hauser, 2002). The proboscis significantly alters the size of the vocal tract, making it longer. Therefore, it can lower the frequency of vocalizations, reducing its correlation with body size (Sanvito et al., 2007b).

The role of the proboscis during visual displays is more uncertain. The proboscis might have a limited visual impact during agonistic interactions, because males generally position themselves one in front of the other (McCann, 1981). Although this is definitely true, males show a large number of postures during displays, including side to side, therefore potentially allowing to use the proboscis as a visual signal. There is no doubt that the proboscis main changes in an individual are related to motivation, as, when a male loses a fight and signal submission, he retreats the proboscis, whereas during the fight is fully expanded (Laws, 1956; Sandegren, 1976). This also offers an evident example of the principle of antithesis (Darwin, 1871), with submissive postures mostly based on the inversion of aggressive signals.

The proboscis is also surely a visual signal of breeding status, as it is related to testosterone level (unpublished data). Indeed, breeding males keep the proboscis fully expanded most of the time during the breeding season, while during the molt they keep the proboscis retracted, as testosterone levels diminish (Griffiths, 1984) and agonistic interactions are avoided (Laws, 1956).

Finally, the proboscis could be a target of female preference. However, evidence for the importance of female choice in elephant seals is scarce, and largely limited to the indirect selection by females inciting males to compete (Cox & Le Boeuf, 1977). Apart from this, female southern elephant seals move little after parturition and have no influence on which male controls a harem (Galimberti et al., 2000). Most of them come into estrus in the same harem where they previously gave birth, and mate with the harem holder, that sires most pups (Fabiani, Galimberti, Sanvito, & Hoelzel, 2004). Males are also very effective at herding females and controlling their movements (Galimberti et al., 2000). All together, female choice seems thus highly constrained by male behavior and it is therefore unlikely to be an important factor in the evolutionary modeling of the proboscis form.

## 4.3 | Perspectives

We showed that it is possible to effectively analyze the variation in proboscis shape by merging field photogrammetry and geometric morphometrics. The potential for applications in field studies of morphological traits related to behavior and reproductive success is clearly great. For instance, after demonstrating high repeatability, one might investigate functional and ecological correlates of proboscis form, as well as assess its variability in relation to male reproductive success. In the population of Sea Lion Island, for instance, this could be done using data collected over more than 20 years (Galimberti & Boitani, 1999).

Despite the success of this first explorative analysis, there is still room for technical improvement. To start, the standardization of the settings for photogrammetry might be increased. We have shown that measurement error was mostly photographic error that was in part due to natural changes in the male posture and/or motivation. Elephant seal males have a predictable pattern of testosterone decrease during the breeding season (Griffiths, 1984). Therefore, a better standardization could be achieved by either taking pictures of males at a specific time since their arrival on the island or including time of tenure as a covariate. Also, the proboscis shows a development during maturation and presents a large individual variation in both size and shape. Therefore, instead of using a cross-sectional sample of males, it would be better to have a longitudinal sample, following the same male along consecutive breeding seasons, as we previously did to study the ontogeny of vocalizations (Sanvito et al., 2008). This might allow to separate individual variation from the population-averaged growth trajectory.

Using 2D photogrammetry, as in this study, has the advantage of relative simplicity but inevitably loses important aspects of variability in a 3D structure (Cardini, 2014). Thus, a better approach would be to apply 3D photogrammetry (Sonnweber et al., 2013). 3D photogrammetry, although sometimes used for estimating seal (Harris, 2012) and sea lion (Waite, Schrader, Mellish, & Horning, 2007) body size, is a rather new field when applied to live animals in the wild. In this respect, a well-studied population of southern elephant seals, such as the one of Sea Lion Island, offers an ideal target for exploring this type

of applications, as during the breed season animals move relatively little, can be easily approached and are present in large numbers.

Finally, it will be crucial to assess the effect of changes in the proboscis shape on vocalizations. As our field protocol includes the routine collection of audio recordings of all breeding males, we can extract data on the frequency structure of the vocalizations (Sanvito et al., 2007b). Therefore, we might be able to examine the correlation between proboscis shape and vocalization frequency structure, hopefully helping to provide an answer to the puzzling questions on the selective pressures behind the evolution of the elephant seal proboscis.

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