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Geometric morphometric analysis reveals that the shells of male and female siphon whelks *Penion chathamensis* are the same size and shape

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ABSTRACT
Secondary sexual dimorphism can make the discrimination of intra and interspecific variation difficult, causing the identification of evolutionary lineages and classification of species to be challenging, particularly in palaeontology. Yet sexual dimorphism is an understudied research topic in dioecious marine snails. We use landmark-based geometric morphometric analysis to investigate whether there is sexual dimorphism in the shell morphology of the siphon whelk *Penion chathamensis*. In contrast to studies of other snails, results strongly indicate that there is no difference in the shape or size of shells between the sexes. A comparison of *P. chathamensis* and a related species demonstrates that this result is unlikely to reflect a limitation of the method. The possibility that sexual dimorphism is not exhibited by at least some species of *Penion* is advantageous from a palaeontological perspective as there is a rich fossil record for the genus across the Southern Hemisphere.

Introduction

Secondary sexual dimorphism can make the distinction of intra and interspecific variation difficult. If males and females of the same species differ significantly in shape or size, the identification of separate evolutionary lineages can be challenging, and taxonomic over-splitting can occur. Sexual dimorphism has been a source of confounding variation in the analysis of extant organisms (e.g. Reskind 1965; Campos 2013; Khorozyan 2014; Underhill and Illiev 2014), and it is especially problematic in palaeontology where genetic and behavioural data are not usually available (e.g. Dodson 1975; Kimbel and White 1988; Huynen et al. 2003). Investigations of morphological stasis and change in the fossil record can identify and focus on single evolutionary lineages if sexual dimorphism is not exhibited.

Although caenogastropod snails are dioecious, sexual dimorphism is understudied within the group. Variation in shell morphology frequently informs gastropod taxonomy (e.g. Reid et al. 1996; Harasewych and Kantor 1999; Kantor 2003; Araya 2013), particularly in palaeontology (e.g. Beu and Maxwell 1990; Frassinetti 2000; Nielsen 2003), and morphometric analyses of shells are increasingly applied at the population and species level (e.g. Tokeshi et al. 2000; Iguchi et al. 2005; Hills et al. 2012; Smith and Hendricks 2013). Historically it was assumed that secondary sexual dimorphism was rare in marine snails (Son and Hughes 2000), but investigations of soft-body

anatomy and shell morphology have since indicated otherwise. Females are typically the larger sex (e.g. Simone 1996; Kenchington and Glass 1998; Kurata and Kikuchi 2000; Son and Hughes 2000; Minton and Wang 2011), although exceptions occur (e.g. Kurata and Kikuchi 2000), and sexual differences in shell shape have been identified in some taxa (e.g. Ten Hallers-Tjabbes et al. 1994; Pastorino 2007; Minton and Wang 2011; Avaca et al. 2013; Mahilum and Demayo 2014).

We investigated whether there is evidence of sexual dimorphism in the shell morphology of the siphon whelk *Penion chathamensis* (Powell, 1938). Siphon whelks are large, benthic true whelks endemic to Australia and New Zealand (Ponder 1973; Powell 1979). The current taxonomy of *Penion* is based upon variation in traditional interpretation of shell morphology and soft-body anatomy (Dell 1956; Ponder 1973; Powell 1979), and there is a rich, intensively collected fossil record for the genus across the Southern Hemisphere (Ponder 1973; Beu and Maxwell 1990; Frassinetti 2000; Nielsen 2003; Beu 2009). *Penion chathamensis* is a large species (shell height 120–215 mm), found in deep water (112–410 m) on the Chatham Rise and Campbell Plateau southeast of New Zealand. Variation in shell morphology of *P. chathamensis* includes adult shell size, length of the siphonal canal, and prominence and persistence of axial ribs on the teleoconch whorls. Such variation is common in true whelks (e.g. Powell 1979; Beu and Maxwell 1990; Nielsen 2003;

Araya [2013]), and is thought to partially reflect environmental plasticity in response to depth, turbidity and substrate (Powell [1927]; Ponder [1971]). In a close relative of *Penion* (Hayashi [2005]), *Kelletia kelletii* (Forbes, 1850), females in mating pairs were found to be on average 13 mm larger than their male partners (Rosenthal [1970]), but no explicit investigation of sexual dimorphism has been conducted within the clade.

To gauge the potential effect of sexual dimorphism on shell variation, we also investigated interspecific variation by comparing shells of *P. chathamensis* to *P. sulcatus* (Lamarck, 1816). *Penion sulcatus* is endemic to New Zealand waters but occurs at shallower depths than *P. chathamensis* (1–165 m), near the North Island and northern South Island coasts. Siphon whelk species appear to exhibit significant intra and interspecific variation in shell morphology, and the differentiation of species is often challenging (Powell [1979]). However, although *P. chathamensis* and *P. sulcatus* are closely related according to mitochondrial 16S RNA gene DNA sequences (Hayashi [2005]), they differ significantly in body size, shell colouration, protoconch morphology and the presence of other shell features such as axial ribs (Powell [1979]).

Using a geometric morphometric approach, we investigated whether male and female *P. chathamensis* differ in shell shape or size, and generated preliminary information about the utility of geometric morphometric analysis applied to *Penion*.

Materials and methods

Specimens used for this study were collected by trawling (20–620 m) or by hand within intertidal depths (1–3 m). Most specimens are held at Museum of New Zealand Te Papa Tongarewa and the National Institute of Water and Atmospheric Research, but additional museum collections were also used (see Supplementary Tables 1–2 and Acknowledgements). A total of 124 *P. chathamensis* shells were sampled across the entire geographic range of the species, including 21 females and 11 males from western Chatham Rise (east of Mernoo Bank), four females from north of the Chatham Islands and two females from the Auckland Islands. The remaining 86 shells came from unsexed individuals. Sexed snails were identified based on the genital anatomy (presence/absence of penis) of live-caught individuals (see Ponder [1973] for description of reproductive anatomy). Since only the Chatham Rise sampling included identified males, and in order to exclude potential interpopulation variation, most analyses were restricted to this group of 32 snails. We also sampled 190 shells of *P. sulcatus* (Lamarck, 1816). Only adult shells that were complete or near-complete with intact edges were included. Maturity was estimated by the presence of at least six teleoconch whorls, thickening of the outer aperture lip and

ascent of the end of the last whorl. Although sexual maturity can occur earlier (Jones [1938]), shell maturity is usually treated as a proxy for adulthood in snails as it indicates when a snail is no longer growing in size (Goodfriend [1986]).

We analysed shell morphology using two-dimensional landmark-based geometric morphometrics, following recommendations listed by Webster and Sheets ([2010]). Shells were mounted in fine-grade silica sand and photographed with the aperture facing upward using a Canon EF-S 600D camera with an 18–55 mm IS II lens (Figure 1). A 50 mm scale bar was included in each digital image. The roll, pitch and yaw were adjusted so that the shells were balanced along the vertical axis (spire to siphonal canal) and the inner lip of the aperture faced directly upward, towards the camera (Figure 1). All positioning, photography and subsequent digitisation were conducted by one person to minimise experimental error
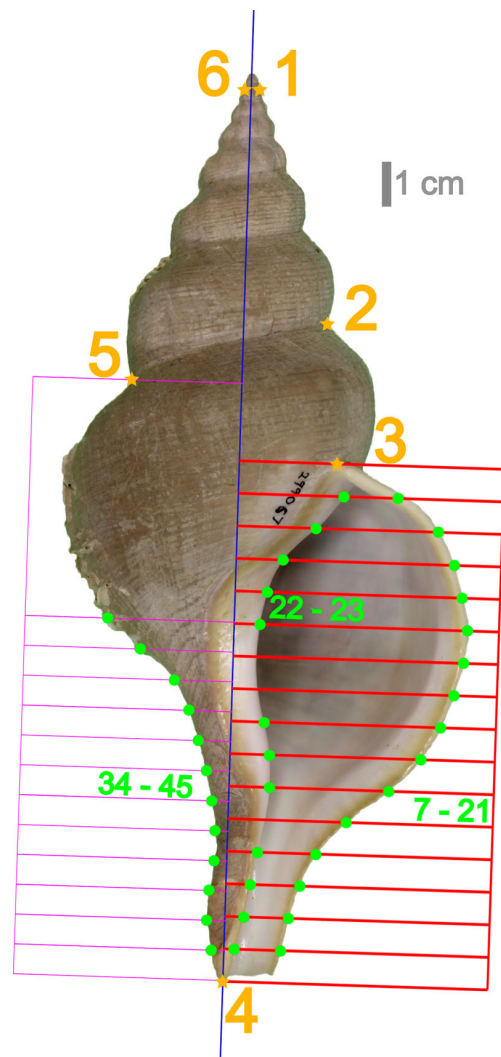

Figure 1. Shell orientation and the configuration of all 45 landmarks (six landmarks [stars], 39 semi-landmarks [circles]) digitised and used for the morphometric analysis of shell morphology in *Penion*.

(Schilthuizen and Haase 2010), which was found to be negligible when investigated (see Supplementary Data). Liveshoot options allowed us to target the camera focus on the aperture and protoconch. For the majority of photographs the camera was mounted on a Kaiser copy stand (RS1, RA-1 arm), but for very large shells it was necessary to use a Compact Action Manfrotto tripod (MKCOMPACTACN) to accommodate large shells within the central field of view using the same camera lens.

Virtual digital combs were aligned to the central axis of the shell and biologically homologous positions such as the end of the siphonal canal were used in Adobe Photoshop CS6 to provide consistent points for digitisation of semi-landmarks (Figure 1). Digital images were organised into thin plate spline (TPS) files using tpsUtil (Rohlf 2013), with the order of specimens randomised to reduce potential experimental bias. Landmarks and semi-landmarks were then identified on each image photographed using a Wacom Cintiq 22HD Pen Display tablet, and then scale-calibrated and slid using tpsUtil, tpsDig (Rohlf 2013) and IMP (Sheets 2014). This yielded X–Y coordinates for points digitised on shells. We used a total of 45 landmarks to digitally summarise shell shape (Figure 1). Six fixed landmarks captured biologically homologous points such as the top of the teleoconch, and 39 semi-landmarks described the inner and outer curves of the aperture and siphonal canal. This number of landmarks was selected after optimisation based on principal component loadings (Supplementary Data). Following the interpretation of Gunz et al. (2005), all of our landmarks (*sensu stricto*) are Type I as defined by Bookstein (1991). Landmarks and semi-landmarks were digitised and scale calibrated using tpsUtil, tpsDig (Rohlf 2013) and the IMP program CoordGen7 (Sheets 2014), yielding X–Y Procrustes coordinates. Semi-landmarks were 'slid' to minimise the effect of the arbitrary placement of points on the curves of interest. Sliding was achieved by minimising Procrustes distances (Bookstein 1997; Zelditch et al. 2004; Perez et al. 2006) using the IMP program Semiland7 (Sheets 2014).

Partial Procrustes superimposition was achieved using MorphoJ 1.06c (Klingenberg 2011), which aligns and superimposes landmarks for all specimens to remove confounding variation due to differences in the size, translation (position) and orientation of objects (Webster and Sheets 2010; Mitteroecker et al. 2013; Monteiro 2013, Polly et al. 2013). Procrustes superimposition is the preferred method when morphological variation is relatively small (Perez et al. 2006). A covariance matrix was generated from the X–Y coordinates of the superimposed landmarks, providing input for principal components analysis (PCA) in MorphoJ 1.06c (Klingenberg 2011). The principal components reflect variation in the shape of objects, and centroid size acts as a proxy for size variation (independent of shape). Statistically significant principal components (PCs) were identified using the broken-stick test on eigenvalues, implemented in the R package vegan 2.2-1 (Jackson 1993; Oksanen et al. 2015). We used PCA ordination to estimate the separation of *a priori* groups (e.g. males and females) using 90% mean confidence ellipses of group means to determine whether groups were likely to overlap. Canonical variates analysis (CVA) was used to test statistically the ability to differentiate these groups, with discrimination success determined using cross-validation scores; the number of individuals correctly assigned to each *a priori* group based on Mahalanobis distance of each individual from group means. This was conducted using either MorphoJ 1.06c (Klingenberg 2011), analysing the original X–Y landmark coordinates or the R package MASS 7.3-26 (Venables and Ripley 2002; R Core Team 2014) using PCs generated from PCA in MorphoJ. For groups with fewer specimens than the number of landmarks used, we used PCA as a dimensionality-reducing method to allow *a priori* groups to be tested with CVA.

We also investigated what groupings could be naïvely identified using the shell morphological data alone, without relying on *a priori* hypotheses based on data such as genetics, taxonomy or geography. To accomplish this we conducted model-based Bayesian assignment analysis using the R package mclust 5.2 (Fraley and Raftery 2002). mclust can analyse both PCs (shell shape) and centroid size (shell size), and it identifies the clustering model that most efficiently explains variation in a dataset without any prior classification of specimens. This is accomplished via an iterative expectation–maximisation (EM) method using Gaussian mixture modelling (Fraley and Raftery 2012). The models tested by mclust differ in the expected distribution of data, as well as the volume, shape and orientation of the covariance matrices generated from observed data (Fraley and Raftery 2012; model parameters listed in Supplementary Table 3). Bayesian information criterion (BIC) scores were used to determine the relative support for competing clustering models. In mclust, BIC scores are multiplied by −1 and therefore higher BIC values indicate stronger support for a given model. Where centroid size was included with PCs for mclust analyses, variables were scaled (using the base function in R) because centroid size is expressed on a much larger numerical scale than PCs. Different numerical scales are problematic for mclust analysis as multiple models tested assume the same variance across all variables or estimated clusters (Fraley and Raftery 2012).

Results

According to CVA, the shape of *P. chathamensis* shells could not be used to successfully differentiate males
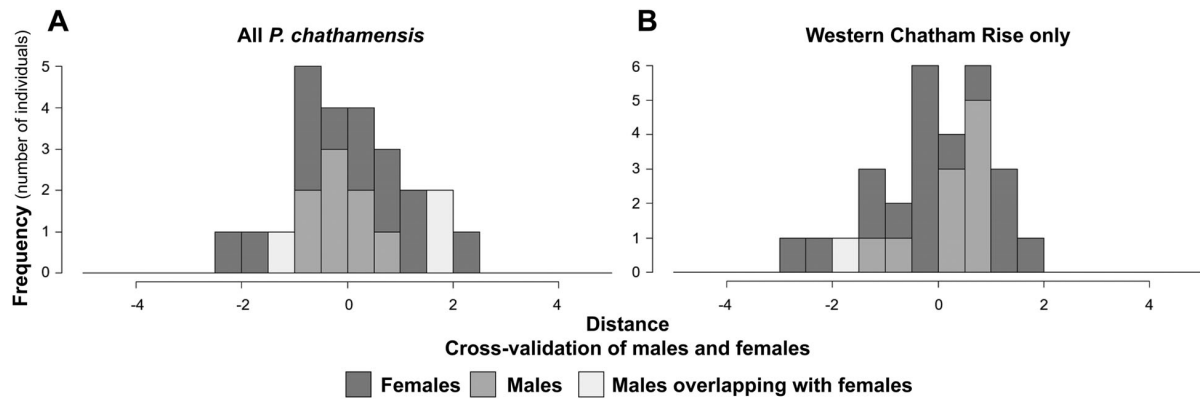
Figure 2. Canonical variates analysis produced using the R package MASS 7.3-26 (Venables and Ripley 2002). Results indicate that the shells of male and female *P. chathamensis* cannot be distinguished, based on the mutual misassignment of individuals (overlapping columns; jack-knifed cross-validation) and the short distance between individuals belonging to each group. The distribution of specimens is shown across all locations (**A**) and for sexed individuals from western Chatham Rise only (**B**). Individuals are coloured according to identified sex: females (dark grey), males (light grey).

from females based on jack-knifed cross-validation scores (Figure 2). More than half of the specimens from Chatham Rise were misassigned to the opposite sex (61.9% of females and 45.5% of males misassigned), and discrimination was similarly poor across the entire species (29.6% females and 54.5% males misassigned). Using PCA with any combination of the significant PCs (variation at PC1 = 39.4%, PC2 = 19.5%, PC3 = 11.4%; PC4 = 7.2%), the similarity in shell shape between

males and females was readily apparent (Figure 3, Supplementary Figure 1). Male and female shape overlapped in morphospace based on 90% mean confidence ellipses. The mean distribution of male and female shape lay close to the mean for the species (*P. chathamensis* as a whole; Figure 3). The mean centroid sizes of males and females were almost identical (male mean = 34.34, SD = 4.57; all female mean = 34.43, SD = 3.99; Chatham Rise only
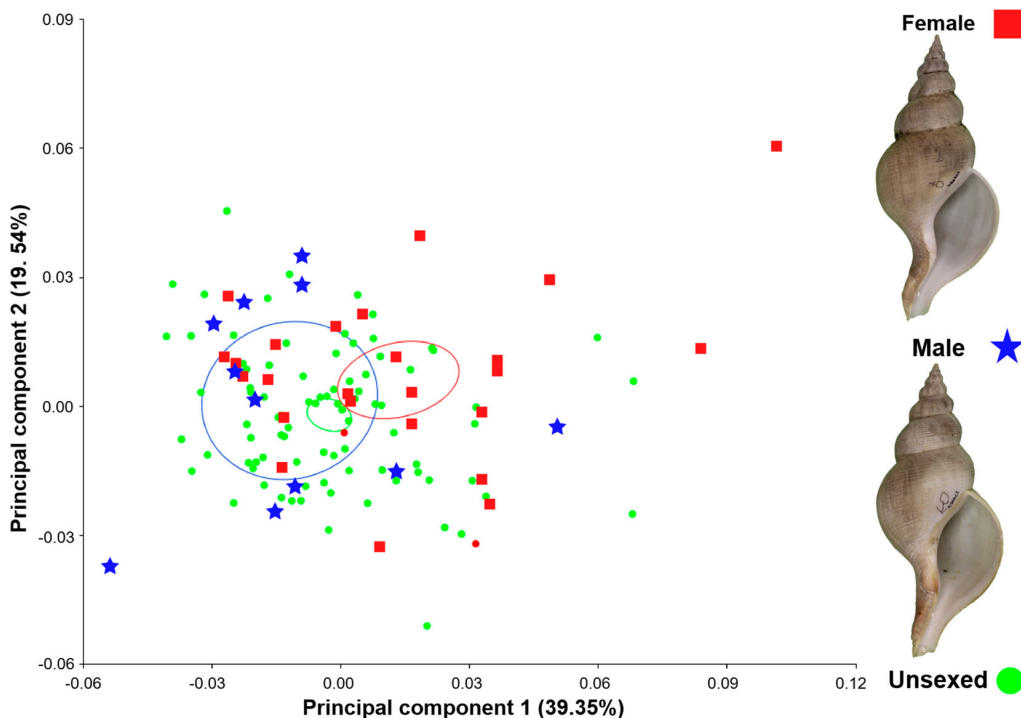

Figure 3. Principal components analysis plot produced using MorphoJ 1.06c (Klingenberg 2011) showing variation among individuals of *P. chathamensis* for principal components generated from geometric morphometric measurements of shells. Principal components 1 (39.35% of variation) and 2 (19.54%) are shown. Females are illustrated as square symbols, males as stars, and shells from unsexed individuals are shown as circles. 90% mean confidence ellipses are illustrated for each group (in matching colouration) and indicate that the means of all three groups are likely to overlap. The sexes were no more distinguishable when the other statistically significant principal components (3 and 4) were included (Supplementary Figure 1).
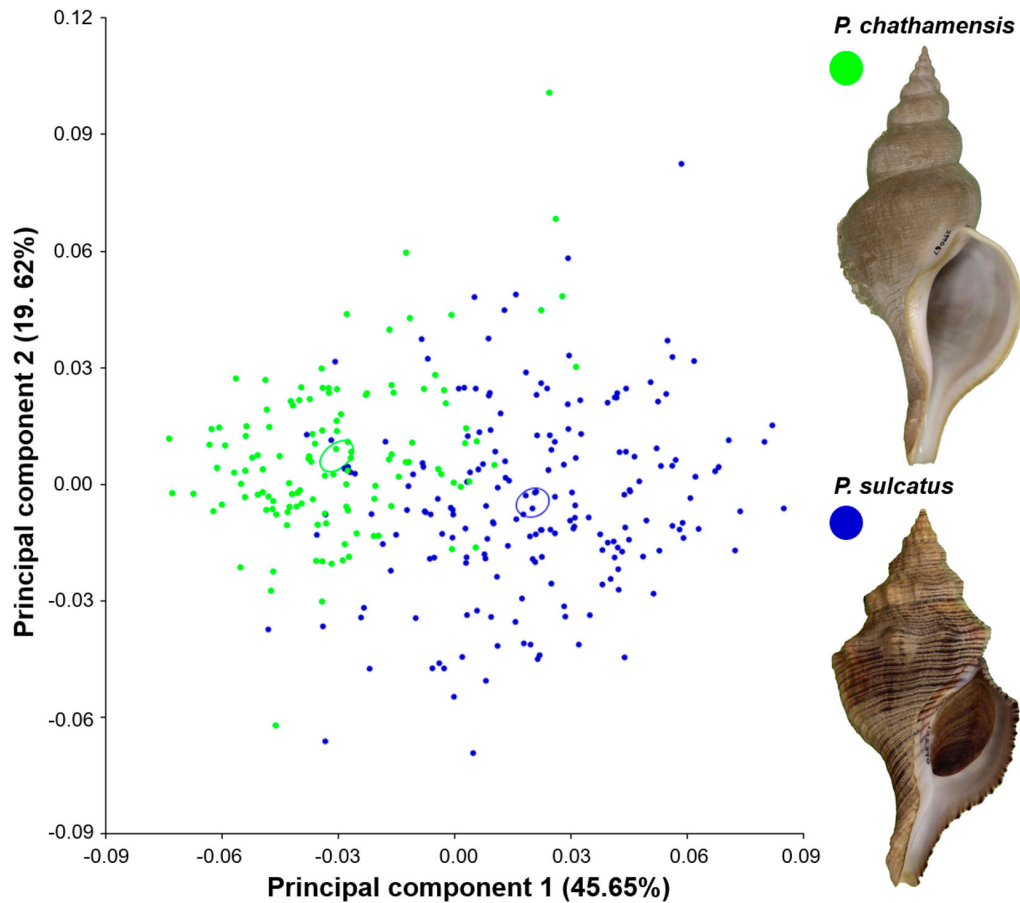
Figure 4. Principal components analysis plot produced using MorphoJ 1.06c (Klingenberg 2011), showing variation among individuals classified as *P. chathamensis* and *P. sulcatus* for principal components generated from geometric morphometric measurements of shells. Principal components 1 (45.65% of variation) and 2 (19.62%) are shown. Specimens classified as *P. chathamensis* and *P. sulcatus* are illustrated as green (light) and blue (dark) circles, respectively. 90% mean confidence ellipses are illustrated for each group (in matching colouration) and indicate that the means of the two species are widely separated in morphospace. The taxa were also readily distinguished using the remaining statistically significant principal component 3.
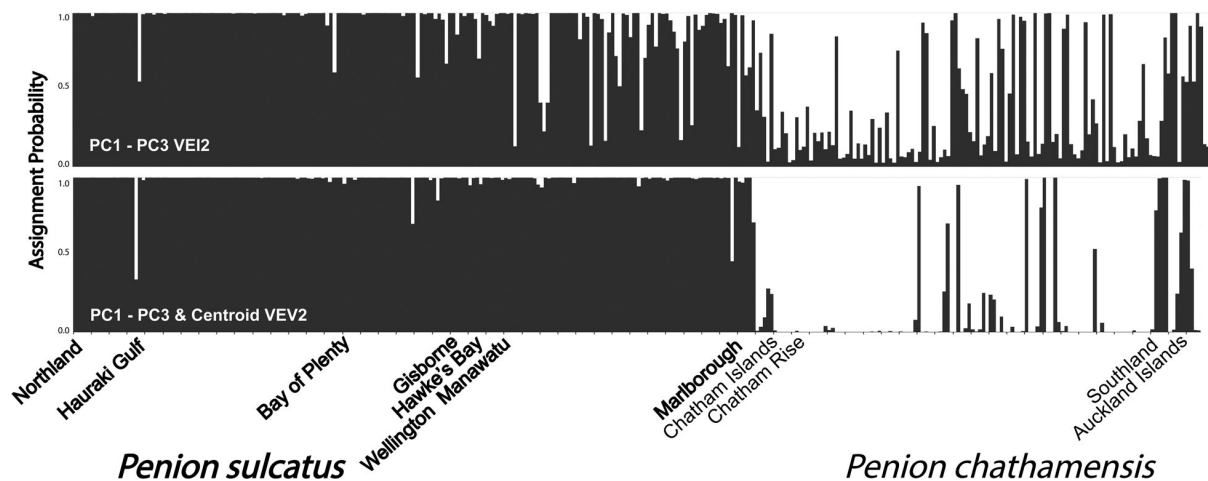

Figure 5. Bayesian assignment probability for each siphon whelk shell belonging to one of the two clusters estimated by modelling shell shape variation of *P. chathamensis* and *P. sulcatus* in the R clustering package mclust 5.2 (Fraley and Raftery 2002). The best supported models are shown for analyses using the statistically significant principal components (1–3) and centroid size with support estimated using the Bayesian information criterion. When centroid size was included, variables were scaled because centroid size is expressed on a much larger scale than principal components. The top plot shows the VEI2 model using PC1–PC3 only, the bottom plot shows the VEV2 model using PC1–PC3 and centroid size. See Supplementary Table 3 for a list of the different parameters used for each clustering model. Species and geographic locations are labelled along the X-axis, with tick marks that indicate every fifth specimen. Clusters identified using the mclust modelling (grey, white) correspond closely with prior species-level identification, despite the analysis not using any *a priori* classification. The inclusion of centroid size mostly increases confidence.

female mean = 35.13, SD = 3.86), indicating that the sexes also do not differ significantly in shell size.

Canonical variates analysis and the ellipses estimated in PCA indicate that males and females are similar, but both analyses rely on the *a priori* classification of individuals (i.e. sex). Neither analysis explicitly attempts to identify the most suitable groupings within the data. We therefore conducted naïve, model-based cluster analysis using mclust. For the sample of sexed individuals from Chatham Rise ($n = 32$), mclust supported only one cluster, whether using the significant principal components alone (PC1–PC4) or with centroid size included (Supplementary Figure 2). This led us to accept the null hypothesis that there are no identifiable groupings within the data; males and females cannot be naïvely distinguished based on shell morphology.

Failure to detect sexual dimorphism in the shells of *P. chathamensis* might indicate that our chosen morphometric landmarks were unsuitable for the detection of relevant morphological variation (a Type II error). To test this, we analysed a dataset containing our sampling of *P. chathamensis* ($n = 124$) and *P. sulcatus* ($n = 190$). Based on CVA using cross-validation scores, *P. chathamensis* and *P. sulcatus* were readily distinguished from one another, with only 0.8% and 1.1% of individuals, respectively, being misassigned to the wrong species. Using any combination of the significant PCs (PC1 = 45.6%, PC2 = 19.6%, PC3 = 8.7%), PCA demonstrates that the two species means are widely separated in morphospace (Figure 4). Some individuals of the two species overlapped in morphospace, but this is to be expected given the highly variable morphology of siphon whelks (Ponder 1973; Powell 1979). Mclust analysis using the significant principal components (PC1–PC3) with or without centroid size, found best support for two clusters of shells (Supplementary Figure 3). These two clusters corresponded closely with the identification of specimens despite the taxonomic classification being derived from traits not captured by our landmarks (e.g. protoconch and axial rib morphology, shell colouration), and accuracy generally improved when centroid size was included (Figure 5). Since mclust was able to naïvely distinguish the species with such high accuracy (Figure 5), despite some overlap in shape space among individuals (Figure 4), we infer that our landmarks are adequate for capturing morphological variation in the shells of siphon whelks. This means that the lack of evidence for secondary sexual dimorphism in *P. chathamensis* likely reflects biological reality.

Discussion

We conclude that sexual dimorphism is not exhibited in shell morphology of *P. chathamensis*, in contrast to results from other Caenogastropoda that suggest sexes differ in shell shape, and that females are usually larger than males (see references in Introduction). It should be noted, however, that some of the previous studies found only weak evidence for sexual dimorphism (e.g. Kenchington and Glass 1998; Son and Hughes 2000). It is also possible that sexual dimorphism is exhibited in a region of the shell not captured by our 2D landmarks (such as the protoconch or the interior of the shell), but this seems unlikely as our landmarks focus on the aperture, which is the end of the generating curve of the shell. Siphon whelks may still exhibit secondary sexual dimorphism in soft-body anatomy. If we assume that other siphon whelk species are similar to *P. chathamensis*, future studies of *Penion* shell variation are likely to be free of the confounding effects of significant secondary sexual dimorphism. This is especially beneficial for palaeontological research where many fossil taxa are known only from single localities or few individuals (Beu and Maxwell 1990; Nielsen 2003; Beu 2009).

Disclosure statement

No potential conflict of interest was reported by the authors.

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