

Geographic morphological variation within and between colour phases in *Coris julis* (L. 1758), a protogynous marine fish species

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Abstract

The possible differences between sexes in patterns of morphological variation in geographic space have been explored only in gonochorist freshwater species. We explored patterns of body shape variation in geographic space in a marine sequential hermaphrodite species, *Coris julis* (L. 1758), analyzing variation both within and between colour phases, through the use of geometric morphometrics and spatially-explicit statistical analyses. We also tested for the association of body shape with two environmental variables, temperature and chlorophyll a concentration, obtained from time-series of satellite-derived data. Both colour phases showed a significant morphological variation in geographic space and patterns of variation divergent between phases. In fact, while the morphological variation was qualitatively similar, individuals in the initial colour phase showed a more marked variation than individuals in the terminal phase. Body shape showed a weak but significant correlation with environmental variables which was more pronounced in primary specimens.

Keywords: geometric morphometrics – spatial analysis – body shape – geographic variation – ecomorphology – sexual dimorphism

Introduction

Phenotypic variation in geographic space can be the consequence of, in addition to other evolutionary factors such as gene flow or genetic drift, variation in environmental variables which, in turn, can influence phenotypic traits both directly and indirectly through trait correlations and interactions with other environmental factors (Lande & Arnold, 1983; DeWitt & Langerhans, 2003; Langerhans, Chapman & Dewitt, 2007). Moreover, the effect of environmental variables on phenotypes can lead to divergent patterns among populations which may or may not be parallel between sexes. That is, given that the majority of the genome is shared between males and females, sexes might show similar patterns of population divergence, but, conversely, differences between sexes (such as in morphology or behaviour) may interact with environmental gradients to produce divergent patterns of phenotypic variation between sexes in geographic space (Hendry *et al.*, 2006).

Body shape variation in fish can reflect ecological and behavioural differences both within and between species, so shape is of particular interest in ecological and evolutionary research (Burns, Di Nardo & Hood, 2009). In particular, intraspecific variation in fish body shape has been related to various factors such as water flow (Langerhans, 2008), feeding habits (Ruehl & DeWitt, 2005), habitat use (Svanbäck & Eklöv, 2003,2004), predation pressure (Gomes & Monteiro, 2008), temperature (Beacham, 1990; Loy *et al.*, 1996) and diet (Wimberger, 1992). Morphometric analyses have been useful in highlighting patterns of stock differentiation in geographic space (Cadrin & Silva, 2005; Cadrin, 2010; Cadrin *et al.*, 2010), even in cases where stock differentiation was undetectable by genetic studies (Levi *et al.*, 2004; Salini *et al.*, 2004). In particular, in the Mediterranean Sea, the morphometric analysis of body shape in *Trachurus trachurus* (L. 1758) has highlighted a structuring in three geographic groups (Murta, Pinto & Abaunza, 2008), a result which is in

44 agreement with analyses using other approaches (Abaunza *et al.*, 2008). However,
45 analyses of body shape variation at the Mediterranean scale are rare as most of studies
46 on body shape focus on a smaller spatial scale.

47 One of the most studied factors affecting body shape in fish is temperature, which typically
48 results in deeper bodies at higher temperatures and more elongated bodies at lower
49 temperatures (Loy *et al.*, 1996; Marcil, Swain & Hutchings, 2006). A recent study on *Danio*
50 *rerio* (Hamilton, 1822) has also evidenced how rearing temperature during the larval phase
51 can have consequences on adult body shape (Georga & Koumoundouros, 2010). While
52 many studies have focused on the plastic response of a single genotype to temperature
53 (phenotypic plasticity), Kavanagh *et al.* (2010) have shown that temperature can induce in
54 *Thymallus thymallus* (L. 1758) genetically-based phenotypic divergence even under
55 conditions of low genetic variation and ongoing gene flow. Temperature can also be
56 correlated with trophic resources: sampling sites with temporally persistent above-average
57 chlorophyll a concentration and below average sea surface temperature have been
58 considered productivity hotspots and have been found to be potentially associated with
59 fisheries resources (Valavanis *et al.*, 2004). Chlorophyll a variability is also associated with
60 feeding intensity in sardine (Garrido *et al.*, 2008). Despite the fact that time series of sea
61 temperature and chlorophyll a data are now easy to obtain as satellite-derived data, such
62 data have not been widely used to study patterns of morphological variation in the field.

63 While patterns of geographic variation in body shape have been studied in both marine
64 and freshwater species, the possible differences between sexes in such patterns have
65 been explored, to the best of authors' knowledge, only in gonochorist freshwater species
66 (Hendry *et al.*, 2006; Langerhans & Makowicz, 2009). In fact, while various studies on the
67 morphometric variation in hermaphrodite fish species exist (Vidalis, Markakis &
68 Tsimenides, 1997; Moran, Burton & Caputi, 1999; Palma & Andrade, 2002,2004), they do
69 not use advanced techniques such as the combination of geometric morphometrics and

70 spatial analysis and they do not test for differences between sexual phases in patterns of
71 geographic variation.

72 The Labridae family is the third largest family of marine fish comprising species which
73 occur worldwide in tropical, subtropical and temperate seas (Parenti & Randall, 2000). The
74 phenomenon
75 of sexual inversion, which is often associated to a change in colour, is very common in
76 labrids (Warner & Robertson, 1978). *Coris julis* (L. 1758) is a small protogynous diandric
77 labrid fish, which is common and almost continuously distributed in most of the
78 Mediterranean Sea. The species, can also be found in the eastern Atlantic European and
79 North African coasts, where it can live in sympatry with its sister species *C. atlantica*
80 (Gunther, 1862) (Parenti & Randall, 2000). *C. julis* is mainly found in rocky coastal areas
81 and *Posidonia oceanica* (L.) Delile, 1813 beds and it is known to have a burrowing
82 behaviour on sandy bottoms at night (Tortonese, 1970)..The species is known to ingest
83 gastropods, crustaceans, bivalves, small fish, polychaetes, echinoderms and benthic algae
84 (Tortonese, 1970; Pinnegar & Polunin, 2000; Kabasakal, 2001) and is considered
85 omnivorous with a preference for animal material (Karachle & Stergiou, 2008). Moreover,
86 Vizzini & Mazzola (2009) have documented for the species a certain degree of variation in
87 the trophic level index among geographically close sampling localities. Guidetti &
88 D'Ambrosio (2004), studying the distribution patterns of *C. julis* and *Thalassoma pavo* (L.
89 1758), another labrid species with similar trophic traits, hypothesized that *T. pavo*, being
90 more markedly thermophilic, outcompetes *C. julis* in shallower waters. *C. julis* juveniles
91 have been repeatedly reported to act as facultative cleaners (Van Tassell, Brito & Bortone,
92 1994; Zander & Nieder, 1997; Zander & Sötje, 2002; Fischer *et al.*, 2007), a behaviour that
93 may be correlated to the presence of a lateral dark stripe (Arnal, Verneau & Desdevises,
94 2006). *C. julis* exhibits two colorations which are so different in morphology that they were
95 originarily described as separate species. The initial phase coloration (also called “primary

livery”) is mainly exhibited by females and consists in a brown upper (dorsal) part of the body and a white ventral part; along with these main features there other sometimes less evident traits like a black and a yellow stripe along flanks. The terminal phase coloration (also known as “secondary livery”) is typical of males and is more colourful with a dorsal portion which is typically green (but also brown or bluish), a ventral white portion and an orange wavy band along flanks with a black elongated mark extending from about the pectoral fin to mid of the body. Initial and terminal colorations are, however, quite variable (Tortonese, 1970; Michel, Lejeune & Voss, 1987; Arigoni *et al.*, 2002). Protogynous sex change is typically accompanied by colour change from the primary to the secondary coloration through a “transitional livery” phase with intermediate colour traits which is relatively short in duration (Bacci & Razzauti, 1957; Bentivegna & Cirino, 1984). Behavioural observations (Lejeune, 1982; Bentivegna & Cirino, 1984) have shown that individuals with primary liveries are sedentary, individuals with secondary liveries are territorial and engage in agonistic behaviours when they meet each other, especially during the reproductive season. Social factors (male/female ratio) have been also shown to induce sexual inversion in the species which lacks of a critical size for sex inversion (Bentivegna & Cirino, 1984). Despite the variation in coloration within livery and among sampling sites (Michel *et al.*, 1987; Laurent & Lejeune, 1988; Arigoni *et al.*, 2002) and the view of *C. julis* as a species that “presents conspicuous morphological variation between populations” (Aurelle *et al.*, 2003), its intraspecific morphometric variation is still largely understudied. The variability in osteological trophic traits in two geographically close populations of the species has been recently assessed (Fruciano, Tigano & Ferrito, *in press a*) but, to the best of authors' knowledge, the scientific literature is lacking of any other morphometric study on the intraspecific morphological variability of the species. Recent work on the species (Fruciano *et al.*, *in press b*) has shown the absence of neutral genetic structuring within the

122 Mediterranean Sea which has been suggested to be the consequence of the high
123 dispersal capabilities of the species, which are, in turn, probably correlated to the long
124 planktonic larval phase (Gordoa, Moli & Raventos, 2000; Raventòs & Macpherson, 2001).
125 The aims of the present study are to test if, despite the absence of neutral genetic
126 structuring, *C. julis* morphology varies in geographic space at the Mediterranean scale and
127 if primary and secondary individuals show different patterns of variation, and also to test
128 for the association with environmental parameters. We expect a significant variation of
129 shape in geographic space for both colour phases with a deeper body at sites with higher
130 temperatures. We also expect that, as already observed in gonochorist freshwater
131 species, patterns of variation in primary and secondary individuals are different because
132 the two colour phases are subjected to different pressures.

133 To test these expectations, a geometric morphometric analysis of body shape variation
134 among nine Mediterranean sampling sites has been performed using, in addition to other
135 methods, spatially explicit analytical tools such as bearing analysis (Falsetti & Sokal, 1993)
136 and trend surface analysis
137 (Krumbein, 1959; Sneath, 1967; Gittins, 1968; Watson, 1971). While Mantel tests of the
138 correlation between morphometric and geographic distances have been used widely in
139 morphometric studies, the use of other spatially-explicit methods with geometric
140 morphometric data is still in its infancy, despite of its advantages. In fact, bearing analysis,
141 which detects the direction in geographic space of greatest correlation of morphometric
142 and geographic distances, has never been used with morphometric data. Similarly, trend
143 surface analysis, which allows to model the variation of one or more dependent variables
144 in geographic space, has been used with geometric morphometric data only recently
145 (Cardini, Jansson & Elton, 2007; Cardini & Elton, 2009; Cardini *et al.*, 2010).

146

Materials and methods

For the present study a total of 223 *C. julis* specimens from 9 different mediterranean populations (Fig. 1; Table 1) was used. Shortly after collection, fish were preserved in 95% ethanol and brought to the laboratory for the acquisition of morphometric data.

Data gathering and dataset preparation

Pictures of the left side of each specimen were taken using a digital camera mounted on a copy stand with an experimental design in which every specimen had two presentations (two pictures) and two digitizations of landmarks for each presentation, for a total of 4 sets of coordinates (such a design was deemed appropriate following the results of a preliminary study of measurement error on a subset of specimens). Throughout the data gathering phase, several measures have been taken to minimize, as much as possible, bias and error: the digital camera was distant from the specimens to reduce the effect of parallax (Mullin & Taylor, 2002), fish were kept straight by running a long needle of appropriate length through the right side of the body (Windsor Aguirre, *personal communication*), all the steps of the analysis have been performed by the same operator, populations were not photographed and digitized at a single session (e.g. all the specimens of a certain population in a single day) but in “rounds” of subsamples to avoid the effects of a potential bias in the way the operator performed his tasks (Windsor Aguirre, *personal communication*); further details on methodological steps are provided by Fruciano (2009).

Twenty points (both landmarks and semilandmarks; Fig. 2) were digitized using the software tpsDig (Rohlf, 2006). The landmark/semilandmark configurations were then subjected to a generalized Procrustes analysis with sliding semilandmarks (Bookstein, 1997) using the software tpsRelw (Rohlf, 2007a) setting ten iterations and the minimization of the squared

Procrustes distance as sliding criterion because this criterion removes all the tangential variation along outlines (Perez, Bernal & Gonzalez, 2006). To reduce both directional and non-directional measurement error, the full dataset comprising four landmark configurations for each specimen has been first subjected to the procedure described in Valentin *et al.* (2008), then the resulting coordinates of each specimen (now adjusted for body arching) have been averaged so to obtain a single landmark configuration for each specimen. Average centroid size measures were also computed for each specimen. Given the marked difference in shape between primary and secondary individuals and following the results of a preliminary study, all subsequent analyses were performed separately for primary and secondary specimens. The program Standard6 of the IMP package (Sheets, 2002) was then used to obtain a dataset in which size-related allometric variation had been removed by regression on centroid size. The resulting configurations of points were then subjected to a principal component analysis (PCA) and PCA scores were used in subsequent analyses.

Shape variation among populations

To analyse body shape variation among populations two Multivariate Analyses of Variance (MANOVA; one for primary individuals and the other for secondary individuals) were performed using STATISTICA (StatSoft Inc) on size-adjusted PCA scores. Pairwise differences between populations were also tested using the permutation procedures on Mahalanobis distances implemented in the CVA module of MorphoJ (Klingenberg, 2011) testing the null hypothesis that the difference was zero. Only sampling sites with at least five specimens were subjected to MANOVA.

To test for differences between colour phases in the pattern of morphological variation between populations, a MANOVA testing for the interaction of livery and site was carried out with STATISTICA on the samples containing at least five specimens of each colour phase.

Analysis of spatial patterns

To analyse the patterns of shape variation in geographic space, three different spatially-explicit approaches were used: Mantel tests (Mantel, 1967), bearing analysis (Falsetti & Sokal, 1993) and trend surface analysis (Krumbein, 1959; Sneath, 1967; Gittins, 1968; Watson, 1971).

The Mantel test (Mantel, 1967) was used to test the null hypothesis that the correlation between pairwise geographic distances and pairwise tangent Procrustes distances between populations was zero. Both geographic distances from geographic coordinates and shortest waterway distances among sampling locations were used. The analysis was carried out both using mean shapes for each livery and each sampling site and using all specimens of a certain colour phase. The test was performed with NTSYSpc 2.2 (Rohlf, 2007b) and PASSaGE 2 (Rosenberg & Anderson, 2011) using 10000 permutations. Bearing analysis is a method of determining the direction of greatest correlation between data distance and geographic distance that has been introduced by Falsetti & Sokal (1993) for the use with genetic data. In bearing analysis, each geographic distance between two points is weighted by the squared cosine of the angle between the line connecting the two points and a reference vector. Then the correlation between the matrix of spatial distances weighted in this way and the matrix of data distances (morphometric distances, in this study) is computed and can also be tested using a Mantel test. The same calculations are then repeated changing the direction of the reference vector and the results can be plotted in a graph where the obtained correlation is depicted as function of the reference direction used for weighting. For the present study, bearing analysis has been performed with

224 PASSaGE 2 using pairwise tangent Procrustes distances among individuals as data
 225 distances. To visualize morphological variation predicted by bearing analysis, the software
 226 tpsRegr (Rohlf, 2007c) was used to regress shape variables on the projection of each
 227 locality on the geographic direction of greatest correlation between morphometric and
 228 geographic distances and to produce three-times exaggerated thin-plate splines of
 229 predicted shapes at each extreme. Bearing analysis has been carried out only on
 230 populations with at least five usable specimens.

231 Trend surface analysis is one of the most powerful tools of spatial pattern analysis
 232 (Legendre & Legendre, 1998) but it has been introduced in the geometric morphometrics
 233 field only recently by Cardini and colleagues (Cardini *et al.*, 2007; Cardini & Elton, 2009;
 234 Cardini *et al.*, 2010). Trend surface analysis consists of the application of polynomial
 235 regression to spatial data. In practice, it is a regression of one or more response variables
 236 (in the case of the present study shape variables) on a polynomial expansion of
 237 geographic coordinates (longitude and latitude, usually centred before the actual analysis).
 238 Being of interest in the present study the broad scale trend of shape variation, a
 239 polynomial of third degree was deemed appropriate following Legendre & Legendre (1998)
 240 and the model $X+Y+X^2+XY+Y^2+X^3+Y^3$ (where X represents the mean-centred longitude, Y the
 241 mean-centred latitude) was used as full model. Three different approaches to find a model
 242 with less parameters were used: the Legendre & Legendre (1998) stepwise method, a
 243 modification of the Legendre & Legendre (1998) method and AICc (Hurvich & Tsai, 1989).
 244 Using the Legendre & Legendre (1998) method, shape variables (principal component
 245 scores) were regressed on all the terms of the full model, eliminating backwards terms not
 246 significant at the 5% probability level one at a time. In case more than one variable was
 247 non-significant in the regression the one to be removed was chosen based on the F-
 248 statistic value (the variable with lowest F-statistic value was removed first). In the modified
 249 version of the Legendre & Legendre (1998) method the only difference was that the

significance level to determine exclusion was 1% ($p=0.01$) so to reduce further the number of variables in the chosen model. Finally, AICc (Hurvich & Tsai, 1989) is a modified version of the AIC (An Information Criterion; Akaike, 1973), adjusted for small sample sizes. The script provided by Al-Subaihi (2002) was used to select the best model using the AICc criterion. All the regressions were performed using the software STATISTICA. For visualization purposes, predicted values for relative warp scores were computed and back-transformed to xy-coordinates, then a five-times exaggerated thin-plate spline of each predicted shape on the consensus shape was produced. Tangent Procrustes distances between all pairs of predicted shapes were then computed with tpsSmall (Rohlf, 2003) and the correlation between the matrices of distances between predictions in primary and secondary specimens was then tested by the means of a Mantel test.

Association of body shape with ecological variables

Time-series of chlorophyll a concentration (October, 16th, 1987-October, 16th 2007; monthly interval) and sea surface temperature (June, 7th, 2004-January, 5th, 2009; weekly interval) at the sampling locations were obtained from satellite data of the American National Aeronautics and Space Administration (NASA). Given that the chlorophyll concentration data were incomplete, only the sampling sites for which more than 40% of the time series was available were used in analyses using this variable, so discarding specimens from Oristano, Pantelleria and Riposto. Arithmetic mean sea surface temperature and geometric mean chlorophyll a concentration (Supplemental material S1) were used as variables choosing geometric mean for chlorophyll a concentration because chlorophyll a distributions tend to be roughly log-normal and it has been noticed that using geometric mean is a better estimator of central tendency (MacFadyen, 1998).

The association of shape with chlorophyll a concentration and sea surface temperature was studied with two-block partial least squares analysis using both ecological variables and also with separate regressions of shape on each ecological variable. Separate regressions of shape variables on chlorophyll a concentration and sea surface temperature were performed using tpsRegr visualizing statistically significant regressions with thin-plate splines of three-times the maximum and minimum observed values relative to reference. A two-block partial least squares analysis using as first block the shape variables and as second block the two ecological variables (standardized to unit variance) was performed with MorphoJ and the significance of the association of morphometric and ecological variables was obtained by the permutational procedure implemented in the program (10000 permutations). Shape variation predicted by partial least squares analysis was visualized with MorphoJ producing "wireframe graphs".

Results

Shape variation among populations

MANOVA rejected the null hypothesis of no difference between population means both in primary (Wilks' Lambda= $F_{234,329.47}=2.029$ $p<0.001$) and secondary (Wilks' Lambda=0.09 $F_{273,482.11}=1.752$ $p<0.001$) specimens. The pairwise comparison among sampling sites using primary specimens is always significant ($p<0.001$). Pairwise comparisons using secondary specimens is always significant ($p<0.001$) except in the case of the comparison between Augusta and Lecce samples. The MANOVA testing for the interaction effect of colour phase and sampling site is also highly significant (Wilks' Lambda=0.0001 $F_{320,536.283}=3.551$ $p<0.001$).

Analysis of spatial patterns

Correlations between shape distances and geographic distances (both as linear distances from coordinates and shortest waterway distances) using average shapes for each sampling site are very low (correlation coefficients ranging from 0.05 to -0.14) and Mantel tests shows that in no case such correlations are statistically significant. On the other hand, correlations are significant when using all specimens separately (that is, the observed Z value is higher than most of the Z values obtained from the permutational procedure; p-value comprised between 0.01 and 0.0004). Also, except in the case of primary specimens and shortest waterway distances (where the correlation is 0.09), the other correlations between morphometric and physical distances using separate specimens are, albeit still low, much higher (range 0.11-0.14) than the ones observed using sampling site means.

The bearing analysis of primary individuals showed that the direction of highest significant correlation between geographic and morphometric distances is 30° (correlation 0.1206, permutational p-value 0.002), corresponding to a direction NE-SW. The bearing analysis of secondary individuals suggests that the direction of highest significant correlation is 111° (correlation 0.1535, permutational p-value <0.001), corresponding to a direction NW-SE. However, the plots of correlations obtained using the various reference directions (Supplemental material S2) show that there are significant correlations also along different directions in geographic space.

The regression of shape variables on projections of sampling localities on the direction of highest correlation explains only 3.74% of variance in primary individuals, 5.14% in secondary individuals. Body shape variation along the directions of highest correlation is depicted in Fig. 3.

The polynomial expansion of latitude and longitude terms performed, as expected, better than a simple regression on latitude and longitude (which would have explained for

primary and secondary specimens respectively 9.73% and 8.11% of variance). Table 2 shows the results of trend surface analysis while Fig. 4 and 5 show shapes predicted from TSA at sampling sites using the model selected by AICc (the other models, while accounting for slightly more variance, produced similar patterns and will not be shown). The mean tangent Procrustes distance between shapes predicted by AICc is 0.009 for primary specimens, 0.006 for secondary individuals. The correlation between tangent Procrustes distances among predictions of primary and secondary specimens is high and significant ($r=0.77$, $p=0.011$).

Association of body shape with ecological variables

Results of the partial least squares analysis are provided in Table 3 and show a weak but significant association between shape and environmental variables. Both in primary and secondary specimens temperature has a positive correlation while chlorophyll has a negative correlation with the first pair of singular axes. Shape changes predicted by the association of shape and environmental variables are depicted in Fig. 6.

Separate linear regression of primary specimens' body shape on sea surface temperature and chlorophyll a concentration are both significant (respectively $p=0.0028$ and $p=0.002$) and explain respectively 5.33% and 5.6% of variance; Fig. 7 depicts shape changes predicted by these two regressions.

Linear regression of secondary specimens' body shape on chlorophyll a concentration was significant using parametric tests ($p=0.037$) but non significant using permutational approaches (0.06) so, given also the extremely low explained variance (1.6%), the regression was deemed non-significant. Regression on sea surface temperature, on the other hand, is highly significant ($p=0.002$) and accounts for 3.9% of variance; Fig. 8 shows shape changes predicted by the regression of body shape on sea surface temperature in secondary individuals.

Discussion

Looking at the results of the MANOVA analyses, it can be said that overall the populations are differentiated from each other.

While the results of Mantel tests should be interpreted with caution as this test has been shown to be affected by both sample size and inequality of sample sizes (Luo & Fox, 1996), the fact that correlations between geographic and morphometric distances are small suggests that in *C. julis* shape differences in geographic space cannot be explained as mere consequence of the distance among sites. The results of bearing analysis show that, along certain directions in geographic space, there is a significant correlation between geographic and morphometric distances, thus suggesting the existence of clines of shape variation. Interestingly, the directions of maximum correlations are different in primary and secondary individuals. The results of trend surface analysis suggest a complicated pattern of variation in geographic space because the polynomial expansion of latitude and longitude terms provides a much better fit to the data (so a simple model describing a linear effect along a direction in geographic space is not completely adequate). The fact that a simple directional gradient in geographic space is not adequate to model shape variation in *C. julis* is also corroborated by the higher levels of explained variance in trend surface analysis compared to variance explained by regression of shape on the projection of sampling sites on the directions of maximum correlation resulting from bearing analyses. While a simple linear effect is not adequate to model shape variation in geographic space, looking at the shapes predicted by trend surface analysis at selected sites for primary specimens, it is possible to discern a pattern. In fact, it can be noticed that the Split population has the most elongated and narrow predicted shape and Naples and Lecce have also an elongated and narrow predicted shape (but not to the extent of the Split sample); all the more southern samples show in general a deeper body. Similarly,

375 Oristano shows, compared to the Mallorca population, a more elongated shape (still not to
376 the extent of the Split sample). Trying to capture an easily explainable general trend, it
377 could be said that at similar longitudes northern populations show a narrower and more
378 elongated body shape, at similar latitudes eastern populations show a narrower body. It is
379 important to remember, however, that such a “general overview” represents an
380 oversimplification of a more complex, statistically significant, pattern at the studied
381 geographic scale. In secondary individuals, it can be said that the pattern is somewhat
382 similar, but less marked, to the one found in primary individuals with Split having narrower
383 bodies than the other samples. While the inclusion of more sampling sites would probably
384 have allowed for a more precise modelling of morphometric variation in geographic space,
385 it is remarkable that all the methods used agree in detecting a variation in geographic
386 space.

387 The analysis of the association between body shape and environmental variables show a
388 weak but significant correlation. In primary individuals, given the different sign of the
389 coefficient on the first singular axis for chlorophyll a concentration and sea surface
390 temperature, the thin-plate spline visualizing shape changes along the first singular axis
391 suggests that, in areas with higher chlorophyll a concentration and lower temperature,
392 individuals have a narrower, more elongated body. Predicted shapes for the separate
393 regressions on chlorophyll concentration and temperature reveal a similar pattern.

394 Considering that high chlorophyll a concentration and low sea surface temperature are
395 associated to productivity hotspots (Valavanis *et al.*, 2004) and that chlorophyll has been
396 shown to be associated to phytoplankton fraction and feeding intensity in sardine (Garrido
397 *et al.*, 2008), it is possible that body shape of primary individuals of *C. julis* might be
398 influenced by productivity and/or correlated variables.

399 In secondary individuals, while the association between shape and ecological variables is
400 significant, the correlation between temperature and the first singular axis is extremely

401 high while the negative correlation of chlorophyll a concentration with the first singular axis
402 is low. Moreover, considering separate regressions, only the regression on sea surface
403 temperature is significant and the predicted shapes show that individuals inhabiting
404 environments with lower sea surface temperature exhibit a more elongated shape while
405 individuals from habitats with higher sea surface temperature exhibit a deeper body, the
406 same general pattern shown by primary individuals in the regression on sea surface
407 temperature. The variation in body shape observed in both primary and secondary
408 individuals might have functional significance. In fact, a more elongated shape minimizes
409 drag and is predicted to be favoured in case of steady swimming, while a deeper body
410 guarantees higher efficiency in case of unsteady swimming with rapid bouts of activity
411 (Walker, 1997; Langerhans, 2008). Variation in *C. julis* body shape might also be affected
412 by the burrowing behaviour which this species exhibits but such an effect should be tested
413 thoroughly. A more elongated shape in environments with lower sea surface temperature
414 might be caused by a variation in the number of vertebrae, which has been shown to
415 increase with latitude in many fish species (Jordan, 1891; McDowall, 2008) and which can
416 have both genetic and phenotypically plastic components (Yamahira, Lankford & Conover,
417 2006; Yamahira & Nishida, 2009). Temperature has been found to have an effect on body
418 shape in various fish species (Beacham, 1990; Loy *et al.*, 1996; Marcil *et al.* 2006; Georga
419 & Koumoundouros, 2010; Kavanagh *et al.*, 2010). Beacham (1990) in a study on
420 *Oncorhynchus keta* (Walbaum, 1792) found that temperature had a marked effect on juvenile
421 morphometric variation and higher rearing temperatures have been found to determine
422 deeper bodies in *Cyprinus carpio* L. 1758 juveniles (Loy *et al.*, 1996). Similarly, Marcil *et al.*
423 (2006) found in *Gadus morhua* L. 1758 that temperature and food abundance had an effect
424 on body shape: individuals reared at higher temperature or food level had a deeper body
425 and a larger head than those reared at the lower temperature or food supply, with the
426 effect of temperature being more pronounced. Marcil *et al.* (2006), noticing that an

increase in body depth has been found to be associated with higher temperature in various fish species, have also suggested that such a response might be general in fish. Wimberger (1992) found that different diets produced different morphologies in two species of the genus *Geophagus* (Teleostei, Cichlidae). In the light of the above-mentioned previous studies on the effect of diet and temperature on body shape, it is possible to hypothesize that in *C. julis* temperature and chlorophyll a concentration have direct and/or indirect effects. In fact, while a direct effect of temperature is easily hypothesizable given that this environmental factor is known to act directly on body shape (Beacham, 1990; Marcil *et al.*, 2006; Georga & Koumoundouros, 2010), it seems more reasonable that the chlorophyll a concentration is associated with some other, presently unknown, variable which causes the variation in shape. The significant effect of chlorophyll a concentration obtained in the present study might also be a mere consequence of its negative correlation with temperature. Moreover, the fact that the association between body shape and the environmental variables studied is weak suggests that there may be other factors affecting the geographic variation of body shape in *C. julis*. Another possible factor affecting body shape variation in geographic space which should be investigated in future studies is the variation of biological communities in geographic space. In particular, *Thalassoma pavo*, being more markedly thermophilic, is believed to outcompete *C. julis* in shallow waters (Guidetti & D'Ambrosio, 2004) and the abundance of *T. pavo* has been shown to decrease at northern sites (Guidetti *et al.*, 2002). It is, therefore, possible that the extent of the competition between *C. julis* and *T. pavo* varies with temperature in geographic space and that this competition drives, at least in part, the morphological variation observed in *C. julis*.

The present study evidences also interesting differences between primary and secondary individuals both in patterns of variation in geographic space and in the association with environmental variables. This is highlighted by the significant interaction between colour

phase and sampling site in MANOVA. Moreover, while the similarity of patterns of geographic variation in primary and secondary individuals is evident from the examination of plots of predicted shapes and the high correlation of morphometric distances between predicted shapes in primary and secondary individuals, the patterns of variation are less marked in secondary specimens (as shown by the smaller average distance between predictions). Moreover, all the chosen models in trend surface analysis account for more variation in primary specimens. This discrepancy might be explained hypothesizing that the secondary specimens are less prone to factors that shape variability at the studied geographical scale.

The fact that in primary specimens both separate regressions on ecological variables are significant while in secondary specimens only the regression on sea surface temperature is statistically significant suggests the hypotheses that secondary individuals are either less sensitive to the studied ecological variables or that they are influenced by these environmental variables in a more complex way, not revealed by the analyses performed. It is possible to hypothesize that the difference between primary and secondary individuals in patterns of variation in geographic space and association with ecological variables is a consequence of a difference between liveries in constraints that determine morphological variation. In fact, while primary specimens are gregarious and, probably, mostly interested in obtaining food resources, secondary specimens are known to establish territories and engage in agonistic behaviours with each other (Lejeune, 1982; Bentivegna & Cirino, 1984) so body shape in secondary individuals might also be influenced by other factors and be the results of more complex trade-offs.

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Figure legends

Figure 1. Geographic locations for the samples used in the study. AU=Augusta, SR, Italy; LE=Porto Cesareo, LE, Italy; ML=Badia de Palma, Mallorca, Spain; MA=Mazara del Vallo, TP, Italy; NA=Capo Posillipo, Naples, Italy; OR=Oristano, Italy; PN=Pantelleria, Italy; RI=Riposto, CT, Italy; SP=Split, Croatia

Figure 2. Landmark (filled circles) and semilandmark positions.

Figure 3. Three-times exaggerated thin-plate splines obtained to visualise results of the bearing analysis in primary (a, solid arrow) and secondary (b, dashed arrow). The splines show the predicted shape at three times each extreme versus the average shape for each livery (used as reference)

Figure 4. Predicted shapes for the trend surface analysis of primary specimens at the sampled locations.

Figure 5. Predicted shapes for the trend surface analysis of secondary specimens at the sampled locations.

Figure 6. Shape change predicted by partial least squares analysis. The figure shows shape change associated to an increase in temperature and a decrease in chlorophyll a concentration. The reference (average shape) is shown in light grey dashed line and empty circles.

Figure 7. Three-times exaggerated splines showing shape changes predicted by separate regressions of shape on chlorophyll a and temperature using primary specimens. a=negative chlorophyll extreme, b=positive chlorophyll extreme, c=negative temperature extreme, d=positive temperature extreme; in all splines the average shape of primary individuals has been used as reference.

Figure 8. Three-times exaggerated splines showing shape changes predicted by the regression of shape on sea surface temperature using secondary specimens. a=negative extreme, b=positive extreme.

S2. Bearing analysis: plot of correlations at different directions. Circles indicate significant correlations. While the bearing analysis to find the direction of highest correlation on which the splines are based has been carried out at 1 degree intervals for good resolution, the plot shows correlations only at 5 degree intervals to produce an easier to understand picture.

Tables

Table 1 Sampling data for each population used in the study

Sample	Sampling time	Environment	Coordinates	Livery		Total
				Primary	Secondary	
Split (SP)	March 2007	<i>Posidonia oceanica</i> beds; a few meters of depth	43°28'N 16°24'E	7	10	17
Naples (NA)	February 2007	Gravel bottom; 10-12 meters of depth	40°46'N 14°12'E	4	8	12
Lecce (LE)	May 2007 and May 2009	Patches of rocky bottom and <i>Posidonia oceanica</i> beds; 8-20 meters of depth	40°14'N 17°52'E	3	29	32
Oristano (OR)	April 2007	Rocky bottom with photophilic algae; <i>Posidonia oceanica</i> beds; 5-6 meters of depth	39°48'N 8°31'E	24	2	26
Mallorca (ML)	June 2007	Rocky bottom with photophilic algae; 9-17 meters of depth	39°31'N 2°39'E	21	19	40
Riposto (RI)	January 2007; March 2007; April 2008	Patches of rocky and sandy bottom; 4-8 meters of depth	37°43'N 15°13'E	26	2	28
Mazara del Vallo (MA)	June 2007	<i>Posidonia oceanica</i> beds; 3-5 meters of depth	37°38'N 12°35'E	14	19	33
Augusta (AU)	May 2008 and June 2008	Rocky bottom with few photophilic algae; 4-10 meters of depth	37°11'N 15°14'E	6	17	23
Pantelleria (PN)	October 2008	Rocks with <i>Posidonia oceanica</i> patches close to a sandy area with <i>Posidonia</i> ; 5-15 meters of depth	36°50'N 11°59'E	1	11	12
Total				106	117	223

Table 2 Results of the trend surface analysis on primary and secondary specimens; X=mean-centered latitudinal term; Y=mean-centered longitudinal term

Primary specimens		
Method	Chosen model	Explained Variance
Full model	$X+Y+X^2+XY+Y^2+X^3+Y^3$	22.61%
Legendre & Legendre (1998)	$X+Y+X^2+X^3+Y^3$	20.33%
Modified Legendre & Legendre (1998)	$X+X^2+X^3+Y^3$	15.82%
AICc	$X+Y^3$	12.86%
Secondary specimens		
Method	Chosen model	Explained Variance
Full model	$X+Y+X^2+XY+Y^2+X^3+Y^3$	17.79%
Legendre & Legendre (1998)	$X+Y+X^2+Y^2+X^3+Y^3$	15.4%
Modified Legendre & Legendre (1998)	$X^2+Y^2+Y^3$	11.21%
AICc	X^2+Y^2	9.39%

Table 3 Results of the partial least squares (PLS) analysis in primary and secondary specimens. The RV coefficient (Escoufier, 1973) is a multivariate analogue of the squared correlation.

Dataset	Correlation between blocks		Results for each PLS singular axis					
	RV	p-value	Axis	% of total covariance	Correlation of scores between blocks	p-value	Correlation of environmental variables with each PLS axis	
Primary specimens	0.1877	0.0008	1	84.689	0.702	0.0012	Temperature	0.6948
			2	15.311	0.448	0.0579	Chlorophyll	-0.7192
Secondary specimens	0.1368	<0.0001	1	78.272	0.574	0.0001	Temperature	-0.7191
			2	21.728	0.352	0.0027	Chlorophyll	-0.6948
							Temperature	0.9906
							Chlorophyll	-0.1367
							Temperature	-0.1367
							Chlorophyll	-0.9906

Figure 1

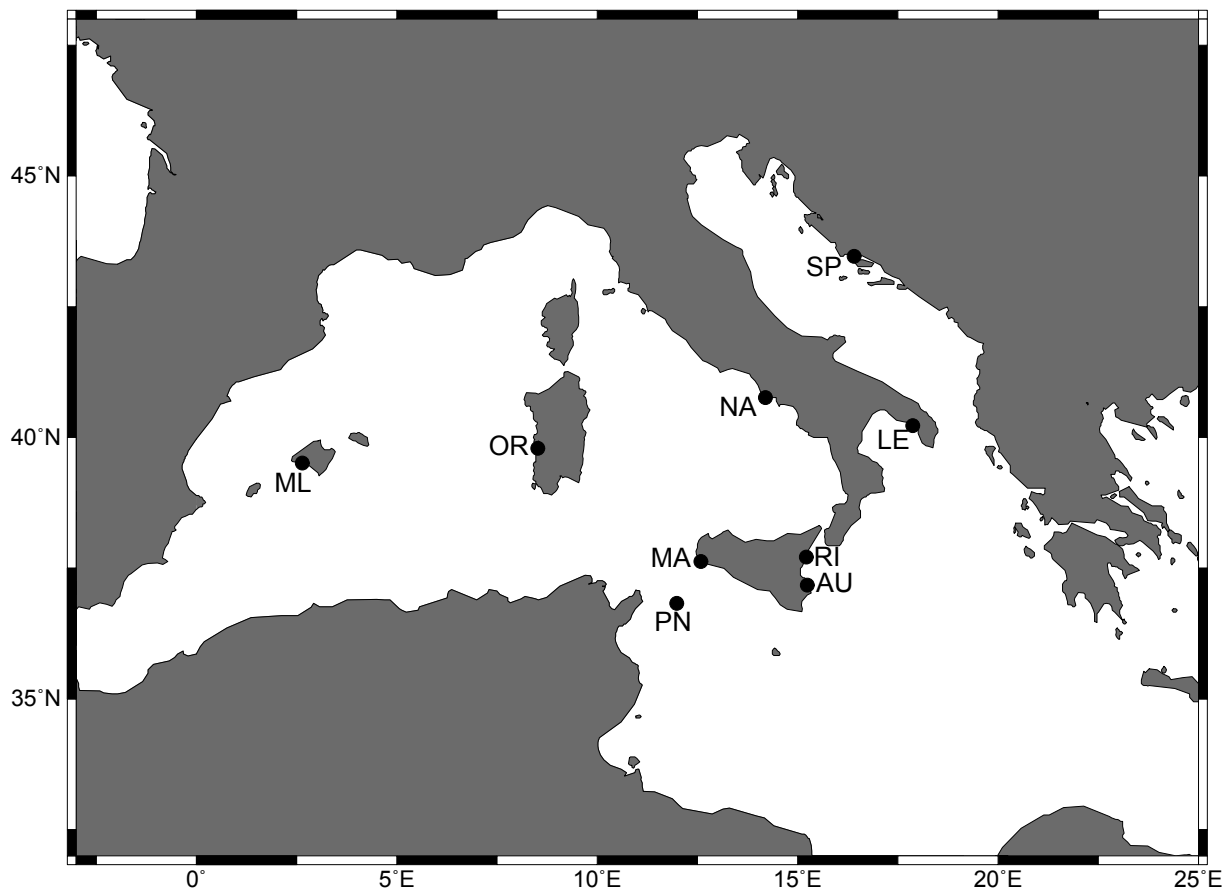


Figure 2

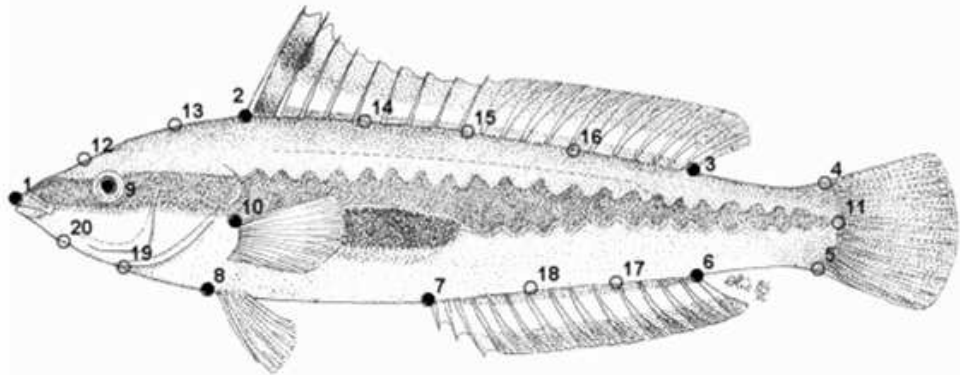


Figure 3

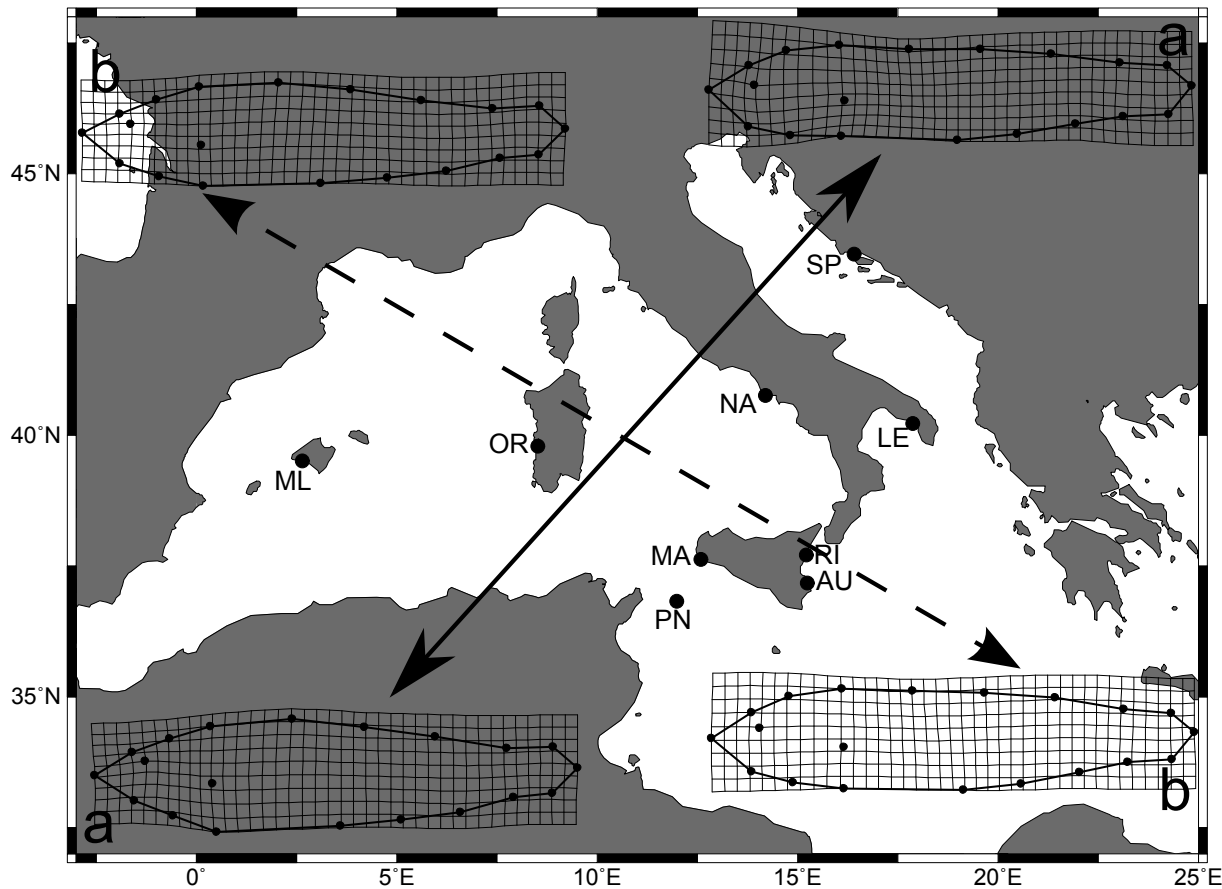
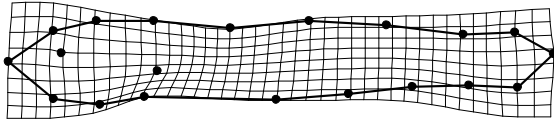
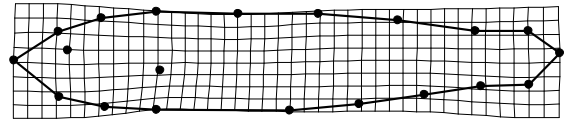


Figure 4

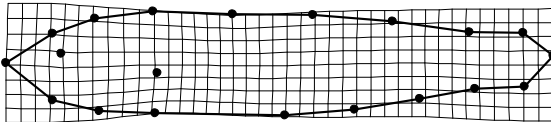
Split



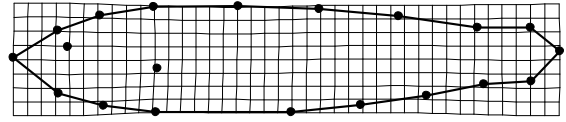
Naples



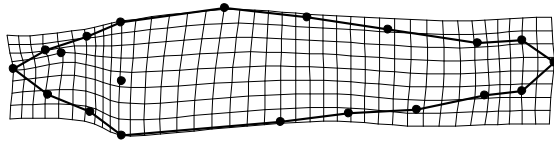
Lecce



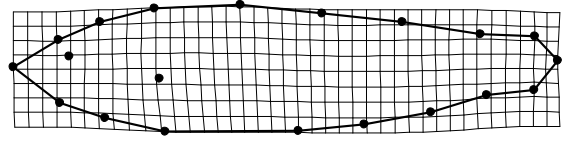
Oristano



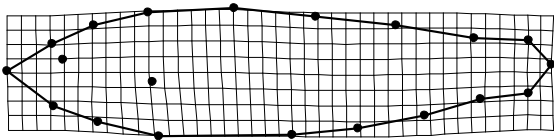
Mallorca



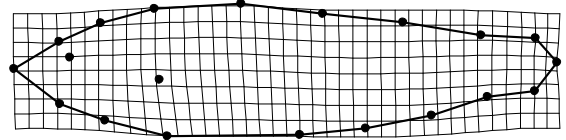
Riposto



Mazara del Vallo



Augusta



Pantelleria

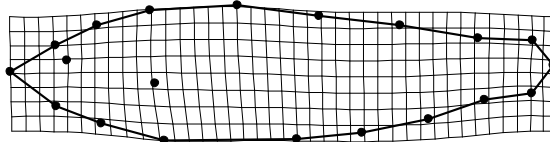
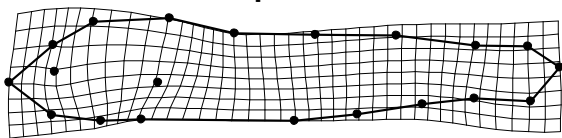
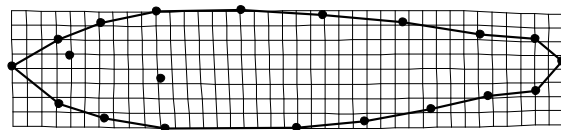


Figure 5

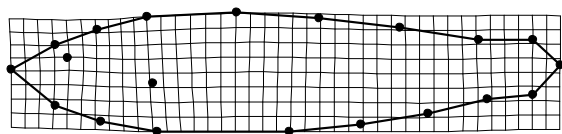
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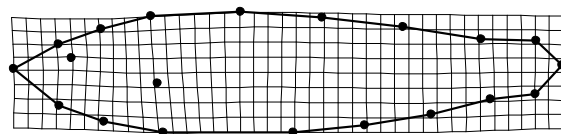
Naples



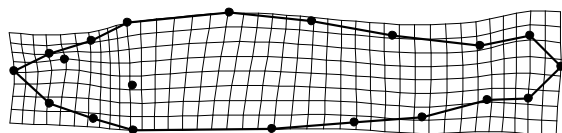
Lecce



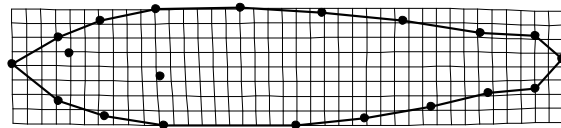
Oristano



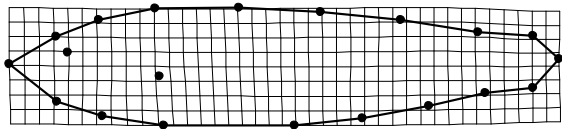
Mallorca



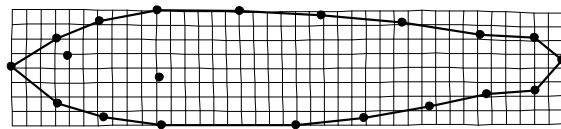
Riposto



Mazara del Vallo



Augusta



Pantelleria

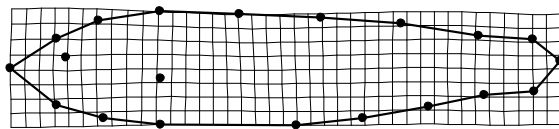
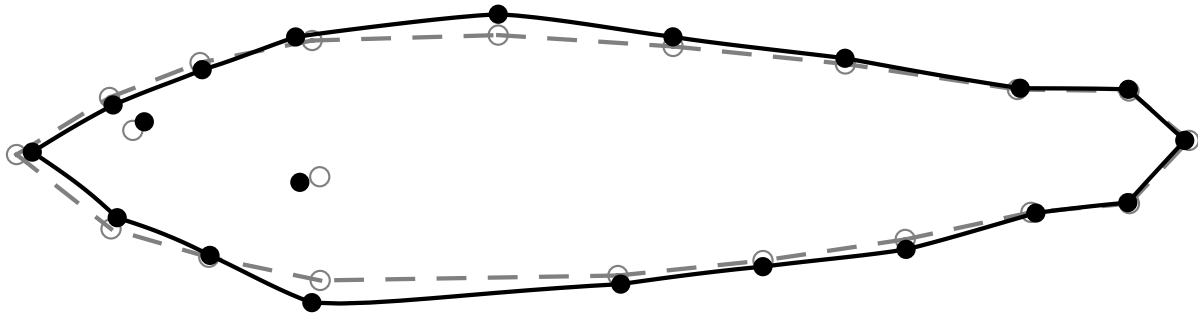


Figure 6

Primary specimens



Secondary specimens

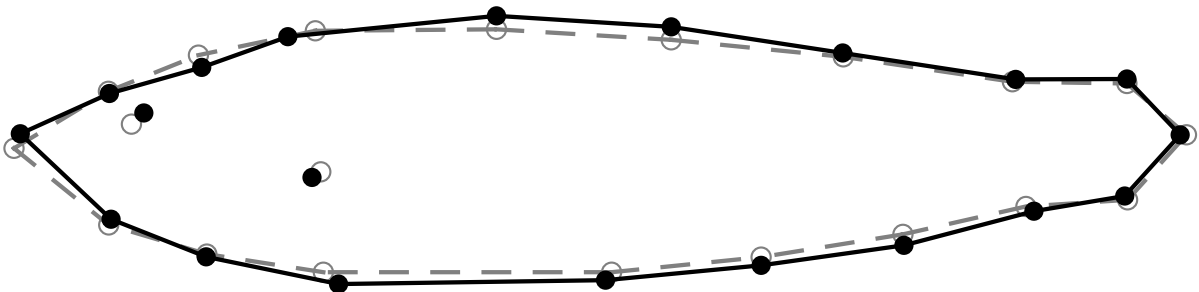
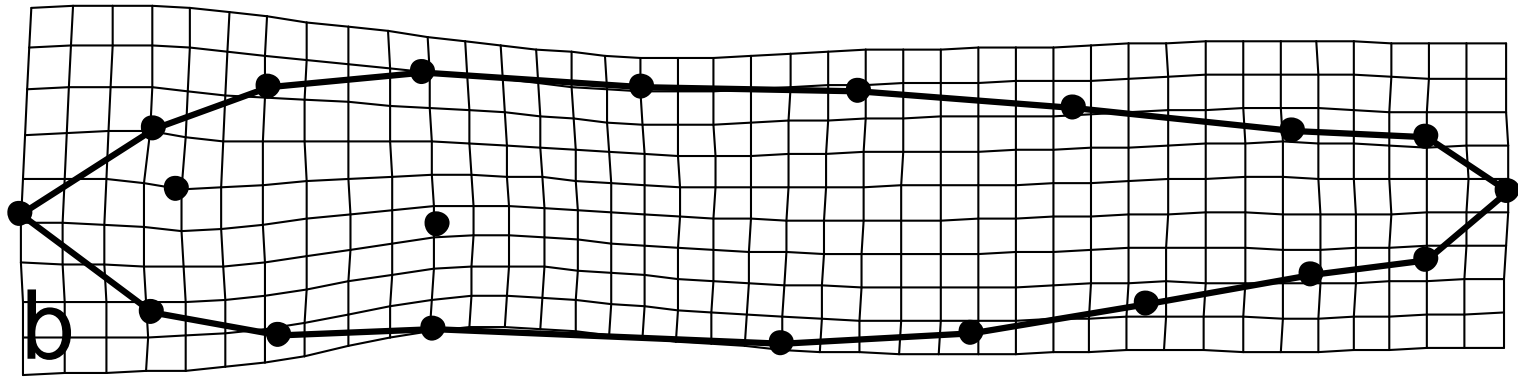
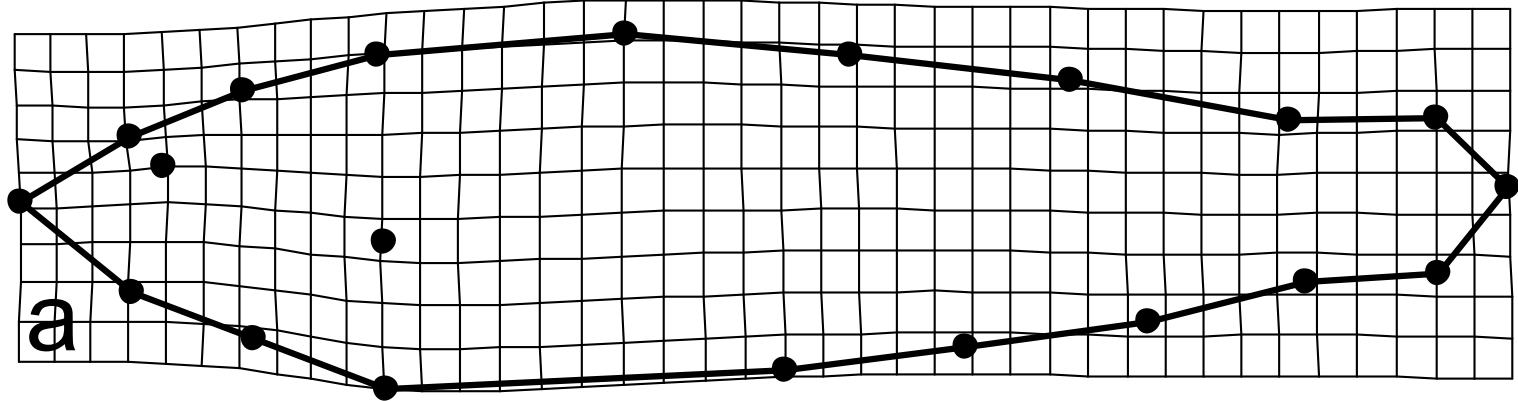


Figure 7

Chlorophyll



Sea surface temperature

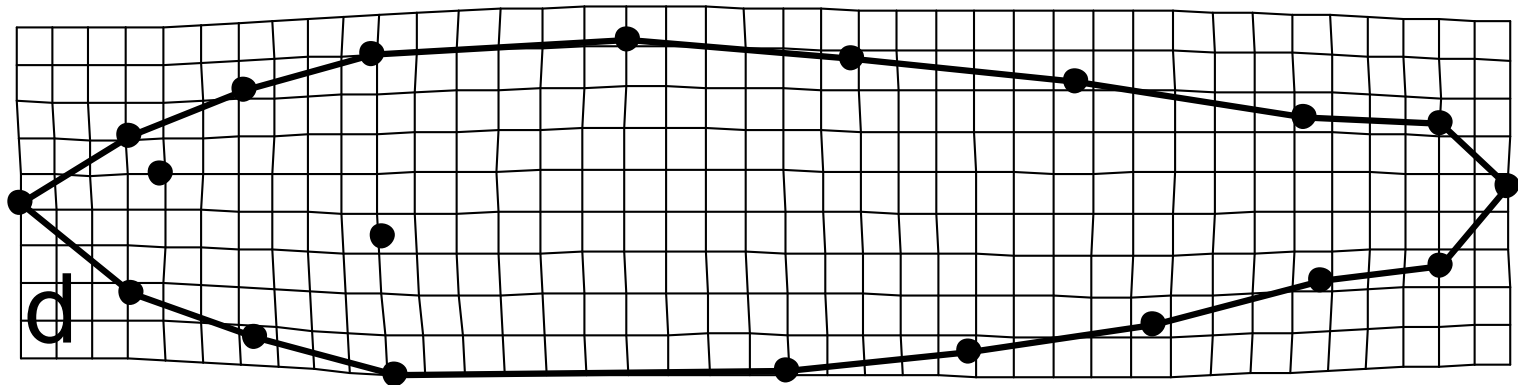
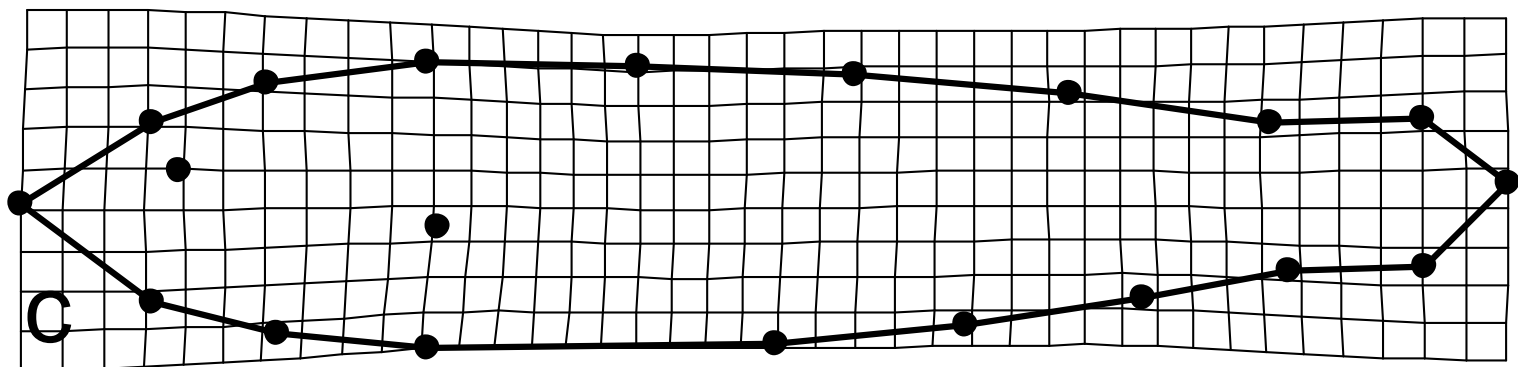
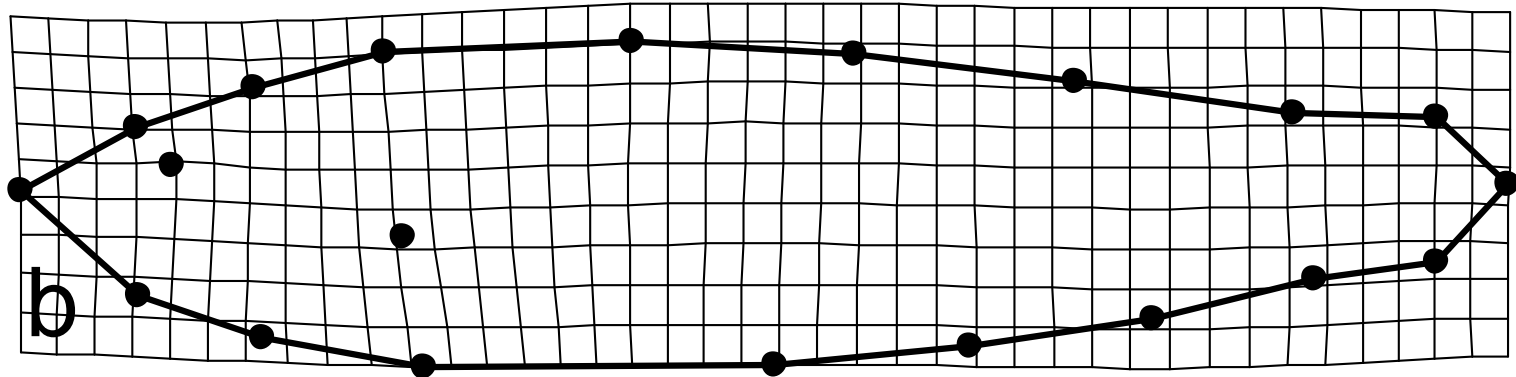
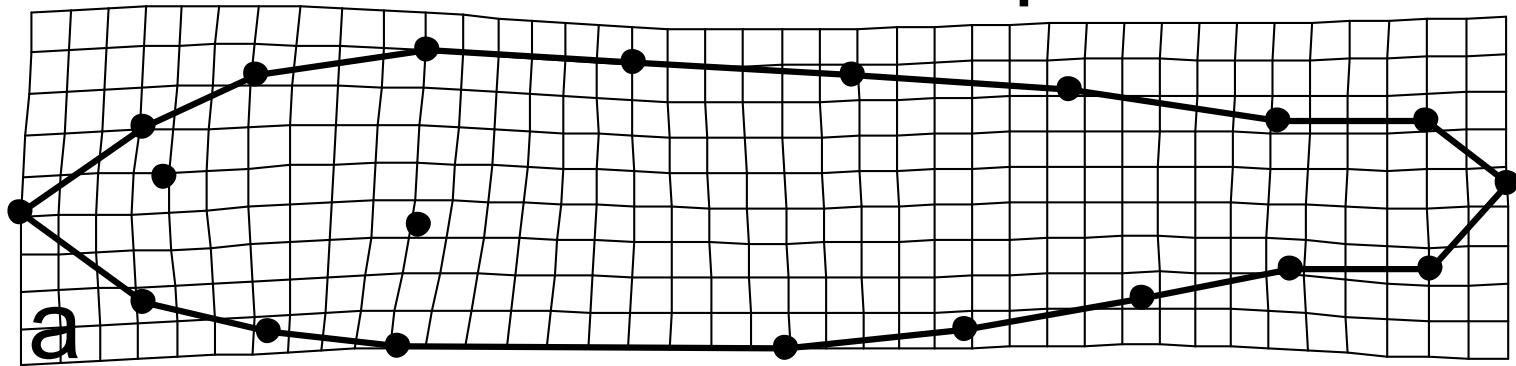


Figure 8

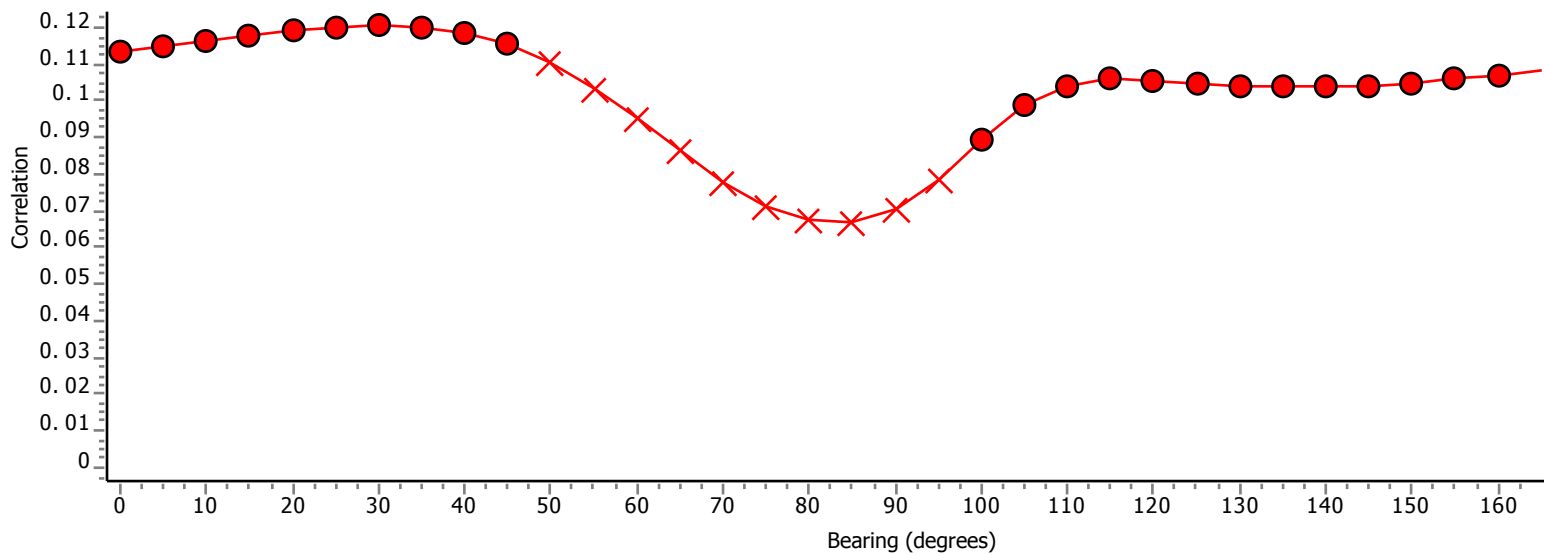
Sea surface temperature



Supplemental material S1: Satellite-derived ecological data.
 Sea surface temperature (SST) in Celsius degrees, chlorophyll a
 concentration in mg/m⁻³

Sampling site	Average SST	Chlorophyll geometric mean
Augusta	20.1825	0.359396216
Lecce	19.38583333	0.379711734
Mazara	19.33291667	0.393831368
Mallorca	19.9975	0.32646652
Naples	19.95833333	0.791435495
Oristano	18.98416667	-
Pantelleria	20.06625	-
Riposto	20.13041667	-
Split	18.41666667	0.501116893

Primary individuals



Secondary individuals

