Environmental Biology of Fishes 94(4), 615-622.

This is the accepted version of the manuscript. The final publication is available at http://link.springer.com/article/10.1007/s10641-011-9968-y

1 2	Body shape variation and colour change during growth in a protogynous fish
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14 15	Keywords: Geometric morphometrics, Labridae, labrids, colour change,
16	protogyny, parallel coordinates
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18	Abstract Protogynous sequential hermaphroditism is very common in marine
19	fish. Despite a large number of studies on various aspects of sequential
20	hermaphroditism in fish, the relationship between body shape and colour during
21	growth in dichromatic species has not been assessed. Using geometric
22	morphometrics, the present study explores the relationship between growth, body
23	shape and colouration in Coris julis (L. 1758), a small protogynous labrid species
24	with distinct colour phases. Results show that body shape change during growth is
25	independent of change in colour phase, a result which can be explained by the
26	biology of the species and by the social control of sex change. Also, during
27	growth the body grows deeper and the head has a steeper profile. It is
28	hypothesized that a deeper body and a steeper profile might have a function in
29	agonistic interactions between terminal phase males and that the marked
30	chromatic difference between colour phases allows the lack of strict
31	interdependence of body shape and colour during growth.

Introduction

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Hermaphroditism is widespread in marine fish where it is present both as simultaneous and sequential hermaphroditism (Shapiro 1979; Warner 1984). While many aspects of sequential hermaphroditism, e.g. gonadal tissutal and morphological variation, body colour variation and size at sex change, in fish have been extensively studied, to the best of the authors' knowledge the relationship between body shape and colour has not been assessed in fish species in which different sexual phases show different colouration. This has happened despite of the fact that the size-advantage hypothesis of sex change and its variations have been extensively explored (Warner 1984,1988; Muñoz and Warner 2003,2004; Warner and Muñoz 2008) and despite of the fact that both body shape feature and colouration are correlated to reproductive or territorial success in a few fish species (Warner and Schultz 1992; Kuwamura et al. 2000). The family Labridae comprises species in which the sexes differ in colour. The first colouration, usually associated to females, is often called "primary livery" while the second colouration, usually associated with males, is called "secondary livery" (Tortonese 1970). Various labrid species are also diandric - that is, there are individuals, called "primary males", which are males but present the primary livery (Reinboth 1967; Warner and Robertson 1978). Coris julis (Linnaeus 1758) is a small protogynous diandric labrid species, which is common along most of the Mediterranean Sea coasts but also inhabits Eastern Atlantic coasts. Recent studies have shown that C. julis lacks genetic structuring at the Mediterranean scale (Fruciano et al. 2011a) but nonetheless shows regional morphometric variation (Fruciano et al. 2011b; Fruciano et al. in press) and that patterns of morphological variation in geographic space can be different between colour phases. Coris julis also shows a certain degree of variability in colour pattern. In fact, primary individuals are known to vary in colour from a brown-based pattern to a reddish pattern as water depth increases and have been found to be greener in Caulerpa taxifolia meadows (Michel et al. 1987; Arigoni et al. 2002). The primary and secondary liveries of C. julis are so different that they were originally described as two different species: Labrus giofredi (Risso, 1810) and C. julis (L. 1758). Behavioural observations (Lejeune 1982; Bentivegna and Cirino 1984) have shown that individuals with primary liveries are sedentary, while 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 shown to induce sexual inversion in the species (Bentivegna and Cirino 1984). Histological aspects of sex inversion in C. julis have been described

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by Bruslé (1987) and Bentivegna et al. (1985), who also noticed a correlation between colour phase and gonadic state. The variation in colouration during sex change, which creates a transitional form with intermediate colouration traits called "transition livery", has been described by Bentivegna and Cirino (1984) who also commented that the changes in colour marks during transition do not always follow the same order. It is unclear, however, if the hormonal changes which are believed to result in colour (and sex) change in this and other labrid species (Reinboth 1975,1988; Reinboth and Brusle-Sicard 1997; Frisch 2004; Ohta et al. 2008) at the same time also cause a variation in body morphology or if the two processes are at least partially independent. In fact, given that certain features of body shape have been shown to be important in territorial interactions among secondary males (Warner and Schultz 1992), it could be expected that a change in sex and colouration would result at the same time in a change in body shape. For this reason, analysing the relationships between size, shape and colouration – as opposed to studying only one trait at a time – can help understanding the relative importance of each morphological feature in the biology of hermaphrodite fish species. Therefore, the aim of this study was to determine if colouration and body shape changes happen simultaneously by using geometric morphometrics coupled with both exploratory and hypothesis-testing statistical tools.

Materials and methods

Dataset preparation

For the present study, a total of 263 *Coris julis* specimens, sampled with fish traps, nets, fishing rods and hand lines at 9 different Mediterranean sites (Fig. 1; Table 1), was used. Fish were preserved in 95% ethanol and brought to the laboratory for the acquisition of morphometrical data. The colour phase of each specimen was determined by visual inspection of the colour marks, identifying as transitional the individuals with colour patterns intermediate between primary and secondary individuals, as described in Bentivegna and Cirino (1984).

Pictures of the left side of each specimen were taken using an Olympus C-3030 digital camera mounted on a copy stand. Each specimen was photographed two times and points were digitized two times for each pictures, obtaining a total of 4 sets of coordinates per specimen (such a design

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was deemed appropriate following the results of a preliminary study on measurement error on a subset of specimens). In the course of the data gathering phase, several measures have been taken to minimize bias and error: the digital camera was relatively distant (495 mm) from the specimens to reduce the effect of parallax (Mullin and Taylor 2002), fish were kept straight by running a long needle of appropriate length through the right side of the body (Windsor Aguirre, pers comm) as to limit dorso-ventral arching, all the steps of the analysis were performed by the same operator, individuals of one population were not all photographed and digitized within the same session but in different ones to avoid any bias in the way the operator performed his tasks (Windsor Aguirre, pers comm); further details on methodological steps are provided by Fruciano (2009). Twenty points (Fig. 2), comprising both landmarks (i.e. homologous points) and semilandmarks (i.e. points which are not homologous but retain positional correspondence), were digitized using the software tpsDig (Rohlf 2006). The landmark/semilandmark configurations were then subjected to a generalized Procustes 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 et al. 2006). To reduce both directional and non-directional measurement error, the full dataset comprising four landmark configurations for each specimen was subjected to the procedure described in Valentin et al. (2008), then the resulting coordinates of each specimen (now adjusted for body arching) were averaged in order to obtain a single landmark configuration for each specimen. Centroid size (the square root of the summed squared distances of each landmark from the center of the form; Bookstein 1989) was also computed for each of the four landmark configuration per specimen and then averaged to obtain an average centroid size per specimen. Statistical analyses

Body shape variation during growth was studied with two approaches: regression on a size measure (centroid size) and exploratory plots of both relative warps and relative warps in sizeshape space (Mitteroecker et al. 2004). Regression of shape variables on centroid size were performed with tpsRegr (Rohlf 2007b), visualizing shape variation with a "wireframe graph" produced by the software MorphoJ (Klingenberg 2011).

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Relative warps in size-shape space were computed as explained by Mitteroecker et al. (2004), adding to the usual shape variables the natural logarithm of centroid size and then performing a principal component analysis (PCA). Plots of individual scores on the first three PC axes were finally obtained with the software STATISTICA (StatSoft, Inc.). To further explore the relationships among size, shape and colour phase in more than three dimensions of multivariate space, we also obtained parallel coordinate plots of relative warps. Using parallel coordinates (Inselberg 1985; Wegman 1990) is an approach that allows a visualization of data with more than three dimensions by avoiding the use of orthogonal axes and substituting them with parallel axes. For each observation (individual) the value of a certain variable is represented by a point on the corresponding vertical axis (each axis represents a variable). The points for each observation on each axis are then usually linked by segments so that each observation (individual) in a sample is represented by a poly-line with vertices on the axes with the position of the i-th vertice corresponding to the value of the i-th variable. While the parallel coordinates technique has been employed in various fields, especially for a visual exploration of data, its use in biology has been rare (Shapley 2004) and, to the best of the authors' knowledge, it has never been used with geometric morphometric data. To check for possible bias in the analysis due to unequal sample sizes or to geographic variation, we also carried out the above-mentioned analyses on a single population (Mallorca) and on a subset of geographically close sampling sites (Augusta, Riposto, Mazara del Vallo, Pantelleria). To quantify the degree of overlap between the portions of morphospace occupied by primary and secondary individuals, we computed the ratio of the convex hull volume (the volume of the ndimensional minimal convex set enclosing a certain set of observation; see Cornwell et al 2006 for an example in ecology) shared by both primary and secondary individuals on the total convex hull volume for primary and secondary primary and secondary individuals. Convex hull computations were performed on the first ten relative warps using the Quickhull algorithm (Barber et al 1996) implemented in Octave (http://www.octave.org/).

Results

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The regression of shape on centroid size was highly significant (Wilk's Lambda 0.27; p<0.001) and explained 23.5% of total variance. Fig 3 depicts body shape changes associated with changes in size and shows a deepening of body during growth and that the relative positions of the eye and the forehead change during growth with the eye closer to the forehead in larger specimens which also show a steeper head profile. In size-shape space, the first relative warp (principal component) was, as expected, highly correlated with the logarithm of centroid size (r= 0.99). The scatterplot of relative warps in sizeshape space (Fig 4) shows that primary and secondary specimens tend to occupy different portions of the multivariate space. Therefore, given that the first principal component has a very high correlation with the size measure and given that successive axes are orthogonal to the first in multivariate space, it can be argued that, if in general terms primary and secondary specimens occupy different portions of the size-shape space, they are different both in size and in shape. However, there is also a certain degree of overlap between the groups of primary and secondary specimens. It is also evident that transitional specimens do not occupy a definite and exclusive portion of the multivariate space - they are scattered across areas where either primary or secondary specimens are prevalent. A similar situation is evident observing the parallel coordinates plot of relative warp scores (Supplementary Fig. S1), which allows visualization of more than three dimensions and where there is a substantial overlap of portions of morphospace occupied by primary and secondary specimens (with a few primary individuals in portions of the space where secondary individuals are prevalent and vice-versa). Observing the parallel coordinate plot, it is also clear that, even considering ten dimensions in the multivariate space of relative warps, the transitional specimens do not occupy a definite cluster. These results do not change restricting the parallel coordinates plot to the quartiles of each livery - that is, considering only the main portions of morphospace occupied by each colour phase – as shown in Supplementary Fig. S2. Convex hull volume computations revealed that 12.37% of the morphospace of occupied by primary and secondary individuals is shared between colour phases. Analyses carried out on a single sampling site and on the subset of geographically close sampling sites gave similar results with significant regression of shape variables on centroid size (Wilk's

Lambda 0.13 and p=0.03 for the Mallorca sampling site, Wilk's Lambda 0.19; p<0.001 for the pooled Sicilian sampling sites) and plots of relative warps and relative warps in size-shape space showing a certain degree of overlap between colour phases (data not shown).

Discussion

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In all the plots of relative warps and relative warps in size-shape space it can be noticed that, while in general primary and secondary specimens occupied different portions of multivariate spaces, there was also a certain degree of overlap (12.37% of convex hull volume). This means that there are typical "feminine" (primary) and "masculine" traits of body shape but not all the specimens of a certain livery conform to this model (that is, there are primary individuals with secondary traits and vice versa). It is also remarkable that in both relative warps and relative warps in size-shape space transitional individuals do not form a definite cluster. While the absence of a definite critical size for colour change - evidenced in our analysis by the overlap of primary, transitional and secondary specimens along the first axis in size-shape space - has already been highlighted in Coris julis (Bentivegna and Cirino 1984), the overlap in body shape is a new finding. Bentivegna and Cirino (1984) proposed that in C. julis there could be individuals that always retain the female phase and showed that sexual inversion can be triggered by social factors (male/female ratio). Social control of sex change is considered common in species with haremic social systems in which dominant males control access to females (Muñoz and Warner 2004). In the light of Bentivegna and Cirino's (1984) observations, our results suggest that shape changes simply happen during growth without regard to colour phase and that livery changes can happen at most of growth stages without interfering with changes in body shape. So, if at a certain point during growth the conditions for livery (sexual) change are present, the individual changes its colour, if not it continues to grow changing its shape without changing its colouration. However, given that specimens with secondary colour pattern are territorial and engage in agonistic interactions with each other (Lejeune 1982; Bentivegna and Cirino 1984), and given that the species is protogynous, most larger specimens are secondary while very large primary specimens are rare. This is in agreement with a size-advantage hypothesis of sex change. In fact, it has been noticed in other protogynous species that the females mate chiefly with larger, older

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males and this factor of strong sexual selection often leads to sexual dimorphism, with males developing specialized structures and colourations for combat and display (Warner and Robertson 1978). A positive correlation of size with reproductive success has been noticed in Halichoeres melanurus males, where it has been hypothesized that size is an important trait in male-male territorial competition while male colouration is important for female mate choice (Kuwamura et al. 2000). Our study also highlights that larger specimens (which are usually secondary in colouration and males) have a deeper body and a steeper head profile, a situation which resembles what happens in other species. In fact, males of many kinds of bony fishes have a prominent nuchal hump on top of the head (Barlow and Siri 1997). In particular, such a nuchal hump has been studied in neotropical cichlids and various hypotheses have been tested to explain this trait (Barlow 1998). However, while Barlow (1998) suggests that in Amphilophus citrinellus the presence of a nuchal hump can be a means of sex recognition, this hardly applies to C. julis due to the striking difference in colour patterns between primary and secondary specimens in this species. Here, it is hypothesized that the deeper body and steeper head profile accompany the striking secondary colour pattern, enhancing it (the vivid colours are probably more visible in a bigger and deeper body) for social display purposes (for instance in mate choice or in agonistic relationships between secondary males). It is worth noticing that in *Thalassoma bifasciatum* terminal males body depth is positively correlated with the success in defending territories from smaller males (Warner and Schultz 1992). A survey of pictures and drawings (Randall and Kuiter 1982; Gomon and Randall 1984; Randall unpubl data) of other Coris species also reveals that a deeper body with growth/terminal livery is easily recognizable in Coris aygula, C. ballieui, C. bulbifrons, C. cuvieri, C. debueni, C. formosa, C. gaimard, C. hewetti and C. sandeyeri. Coris aygula and C. bulbifrons also develop a nuchal hump similar to the one found in cichlid species. These findings suggest that the pattern described here is common to other labrid species of the genus and might represent a new avenue for research on sequentially hermaphroditic fish species. In fact, it is possible that the pronounced difference in colour between primary and secondary specimens observed in C. julis allows the observed decoupling of body shape and colour phase, leaving body shape to be a useful but not strictly necessary "enhancer" of the function of colour. Alternatively, body shape might be more important in species where chromatic differences between liveries are less pronounced; in these cases, colour and body shape changes during growth might be more strictly linked. The relative importance of

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colouration and body shape in aggressive and/or display behaviour of secondary individuals will need to be more thoroughly tested in C. julis and similar species. **Conclusions** C. julis shows a significant allometric variation in body shape which results in deeper bodies in larger individuals. While on average secondary individuals possess a deeper body compared to primary individuals – a common occurrence also in other species of the genus - body shape in C. julis changes during growth without being constrained by colour phase. For this reason, it can be argued that secondary individuals have, on average, a deeper body, just because they are larger and that the hormonal changes which result in sex and colour change seem not to have effect on body shape. Finally, our study also shows that parallel coordinate plots can be a useful exploratory tool when dealing with geometric morphometric data. Acknowledgements The authors would like to acknowledge the precious contribution of F. James Rohlf who provided many useful suggestions at multiple stages. Enrico Arneri, Ferdinando Boero, G. Andrea de Lucia, Paolo Domenici, Maria Ghelia, Paolo Guidetti, Miquel Palmer, Sergio Ragonese, Alfonso Toscano and Nedo Vrgoc kindly helped in obtaining specimens used in the analysis. References Arigoni S, Francour P, Harmelin-Vivien M, Zaninetti L (2002) Adaptive colouration of Mediterranean labrid fishes to the new habitat provided by the introduced tropical alga Caulerpa taxifolia. J Fish Biol 60:1486–1497. Barber CB, Dobkin DP, Huhdanpaa HT (1996) The Quickhull algorithm for convex hulls. ACM T Math Software 22(4):469-483. Barlow GW (1998) Sexual-selection models for exaggerated traits are useful but constraining. Am

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Figure captions 354 355 Fig. 1 Geographic locations for the samples used in the study. AU=Augusta, SR, Italy; LE=Porto 356 Cesareo, LE, Italy; ML=Badia de Palma, Mallorca, Spain; MA=Mazara del Vallo, TP, Italy; 357 NA=Capo Posillipo, Naples, Italy; OR=Oristano, Italy; PN=Pantelleria, Italy; RI=Riposto, CT, 358 Italy; SP=Split, Croatia 359 360 Fig. 2 Position of the points used for the analysis of body shape. Filled circles represent 361 landmarks, open circles semilandmarks 362 363 Fig. 3 Change in shape associated with change in size represented with a "wireframe graph" 364 produced by the software MorphoJ. The dashed grey line represents a reference shape, the black 365 solid line represents the change in shape relative to the reference as the body size increases 366 367 Fig. 4 Three-dimensional scatterplot of the first three relative warps (RW) in size-shape space; 368 the scale of the axes has been distorted deliberately to visualize the patterns on RW2 and RW3. 369 The plot shows a considerable overlap of primary and secondary individuals, as well as the lack of 370 a definite region for transitional specimens 371 372 Supplementary Fig. S1 Parallel coordinate plot of the first ten relative warps for all the studied 373 specimens. Yellow=primary, blue=transition, pink=secondary. The plot shows a considerable 374 overlap of the morphospace portions occupied by each colour phase 375 376 Supplementary Fig. S2 Parallel coordinate plot of the first ten relative warps. For each colour 377 phase only the quartiles are shown to highlight the degree of overlap among groups. 378 Yellow=primary, blue=transition, pink=secondary. The plot shows that the overlap in 379 morphospace occupation of primary, transitional and secondary individuals is not caused by a few 380 outliers but it is evident even considering only the quartiles of the relative warps of each colour 381 phase 382

Table 1 Sampling data for each population used in the study

Sample	Sampling time	Environment	Coordinates	Livery			Total
				Primary	Transition	Secondary	
Split (SP)	March 2007	Posidonia oceanica beds; a few meters of depth	43°28'N 16°24'E	7	9	10	26
Naples (NA)	February 2007	Gravel bottom; 10- 12 meters of depth	40°46'N 14°12'E	4	21	8	33
Lecce (LE)	May 2007 and May 2009	Patches of rocky bottom and Posidonia oceanica beds; 8-20 meters of depth	40°14'N 17°52'E	3	1	29	33
Oristano (OR)	April 2007	Rocky bottom with photophilic algae; Posidonia oceanica beds; 5-6 meters of depth	39°48'N 8°31'E	24	4	2	30
Mallorca (ML)	June 2007	Rocky bottom with photophilic algae; 9-17 meters of depth	39°31'N 2°39'E	21	2	19	42
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	Posidonia oceanica beds; 3-5 meters of depth	37°38'N 12°35'E	14	2	19	35
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 Posidonia oceanica patches close to a sandy area with Posidonia; 5-15 meters of depth	36°50'N 11°59'E	1	1	11	13
Total				106	40	117	263

Fig. 1

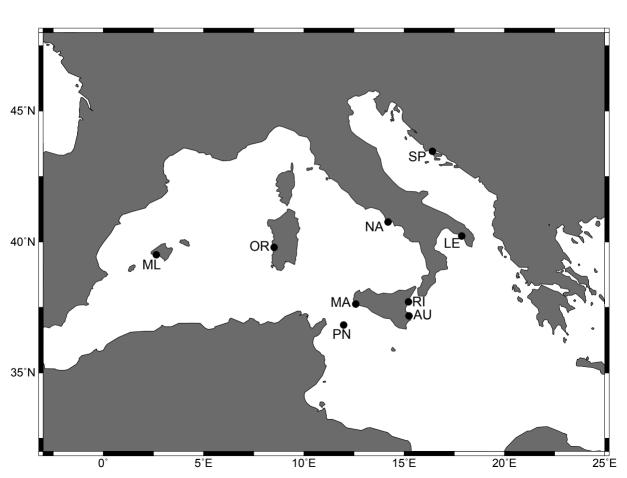


Fig. 2

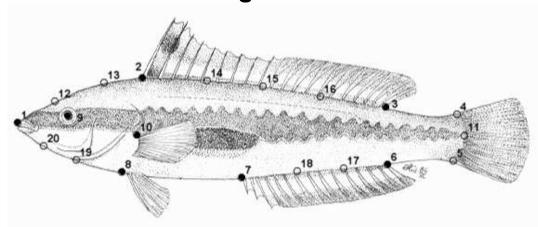


Fig. 3

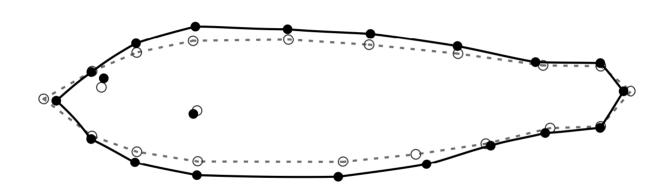


Fig. 4

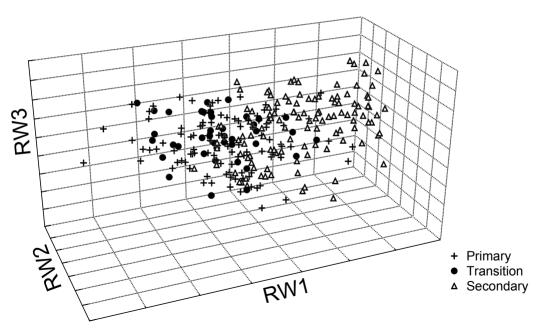


Fig. S1

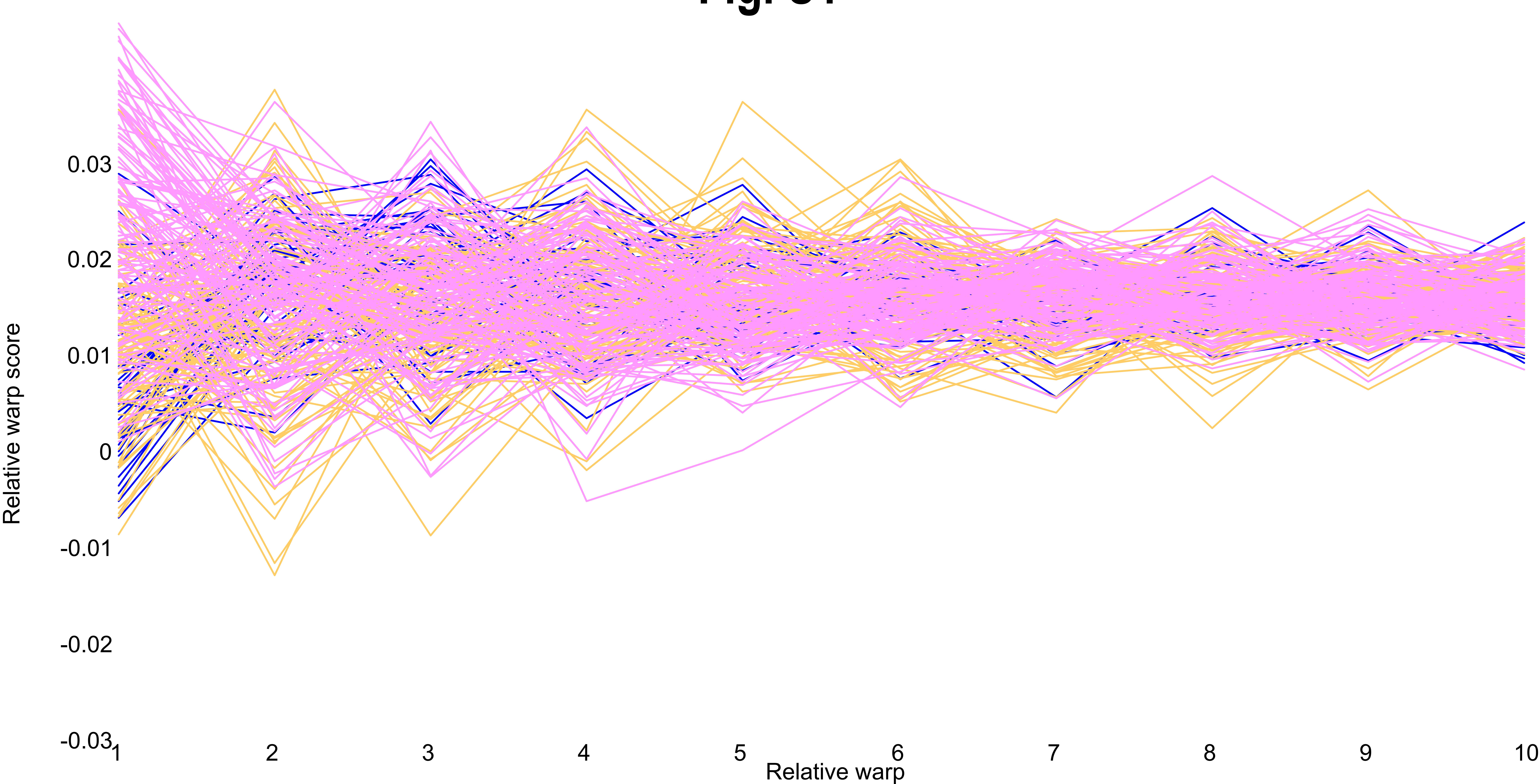


Fig. S2

