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### Article

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# 1 Exploring fluctuating asymmetry in two recovering populations of the Eurasian otter

2

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## 11 Abstract

12 The Eurasian otter is a wide-ranging semi-aquatic mammal that underwent a significant population  
13 decline in the last century, leading to local extinctions, reduction and fragmentation of populations.  
14 Populations exposed to both external and internal stress may exhibit Developmental Instability (DI).  
15 Factors contributing to DI include inbreeding depression, population bottlenecks, habitat loss, and  
16 exposure to pollution. We analyzed fluctuating asymmetry (FA) as a proxy of DI in two European  
17 otter populations that experienced a major decline in the '90. We recorded 30 3D landmarks on 98  
18 skulls from Sweden (n=69) and the United Kingdom (UK, n=29), and compared the degree of FA  
19 both between populations and before, during and after the decline in each population. We found a  
20 higher FA in UK compared to Sweden. The Individual Signed Asymmetry Index differed significantly  
21 across time only for the UK population, displaying higher values during the decline phase. FA in UK  
22 population was attributed the impact of PCBs pollution and to subsequent bottleneck. Our study  
23 suggests that habitat loss, pollution, and limited gene flow may contribute to increased FA and DI in  
24 declining otter populations, highlighting the need for continued investigation into specific stressors  
25 affecting otter populations.

## 26 Introduction

27 The Eurasian otter (*Lutra lutra*) is a semi-aquatic mammal distributed across Europe, Asia and North  
28 Africa [1]. This species can live in a wide variety of aquatic habitats including lakes, rivers, and  
29 coastal environments [1]. In recent decades, the species has undergone a drastic population decline  
30 in parts of its range [2]. Multiple factors were involved in the decline, including hunting for fur,  
31 persecution by fishermen, roadkills, habitat fragmentation and pollution [2]. However, thanks to legal  
32 protection and habitat restoration, the species is now recovering in its former range [2,3] and at  
33 present it does not appear to be threatened by climate change [4,5].

34 Populations exposed to both external and internal stress may present Developmental Instability (DI).  
35 DI implies the inability of an individual to produce the a specific developmental outcome, generating  
36 developmental "noise" that can result in congenital disorders [6,7].

37 This process is driven by external or internal factors of stress that can affect the developmental  
38 pathway of phenotypic structures expected by the genotype [8]. Multiple factors can result in  
39 developmental instability, such as inbreeding depression [9–11], population bottlenecks [12,13],  
40 urbanization [14], habitat loss [15], and exposure to pollution [16]. For example, Loy et al. [11] found  
41 that the isolated population of the Apennine brown bear (*Ursus arctos marsicanus*) showed high  
42 levels of DI as a consequence of inbreeding depression, while Caccavo et al. [15] observed that  
43 habitat loss can lead to high levels of DI in the South American water rat (*Nectomys squamipes*).

44 In bilateral skeletal structures of vertebrates such as the skull, DI can produce asymmetry randomly  
45 skewed towards the right or the left side, a phenomenon known as fluctuating asymmetry (FA)  
46 [17,18]. Thus FA identifies and quantifies the degree of random deviation from bilateral symmetry  
47 measure [19]. As this method is a reliable, non-invasive, and cost effective, it is commonly used to  
48 detect developmental instability method [20]. FA is distinct from directional asymmetry (DA; [18]) as  
49 the latter is characterized by directional asymmetric changes that always favour one side, and are  
50 commonly driven by specific adaptive pressures. An example of DA is the asymmetrical skull of  
51 dolphins [21].

53 Previous work using traditional linear morphometrics identified high levels of FA in the skull  
54 of Danish populations of the Eurasian otters due to environmental stress  
55 [22, 23]. Here, we explore the variation of FA in the skull of two  
56 Eurasian otters populations  
57 showing similar latitudinal morphometric gradient (Sweden, and UK [22,23]).

58 Specifically, we: *i*) investigated size and shape variation and the degree of FA in otters from Sweden  
59 and UK using a 3D geometric morphometrics (GM) approach, and *ii*) explored if and how FA has  
60 changed over the last decades in response to specific drivers.

61 We expect higher levels of FA in both populations as a consequence of population decline,  
62 habitat loss, and exposure to pollutants.

## 63 **Methods**

### 64 *Sample collection*

65 We produced 3D models for 98 skulls of adult otter specimens housed in three museum collections  
66 (Natural History Museum, London UK (NHMUK), National Museums of Scotland (NMS) and Swedish  
67 Museum of Natural History (SMNH). All specimens belong to population from the UK (n=29; M=18,  
68 F=10, NA=1) and from Sweden (n=69; M=40, F=29). Information on sampling area, year of  
69 collection, age, and sex were extracted from museum labels and are available in Supplementary  
70 Material (S1); map samples location is shown in Supplementary Material (S2).

71 The age of specimens was also checked by examining the status of cranial sutures (i.e.,  
72 frontoparietal, zygomatic arch) and permanent dentition [24]. Years of the collection were grouped  
73 into three time series based on the start, ongoing, and end of the decline of otters populations in  
74 Europe [2] as follows: before  
75 (recovery).

76 Due to limited availability of specimens with complete information on sex, age, locality, and year  
77 of collection from the same region for the three sampling periods, each time period was represented  
78 by samples from one or more areas. Specifically, for UK the 'Before' period included samples  
79 from England (n = 2) and Scotland (n = 4); the 'During' period from Scotland (n = 6) and Shetland (n  
81 = 9); the 'After' period from Wales (n = 8). As for Sweden, the 'Before' period included samples from  
82 South (n = 4), the 'During' and 'After' periods samples from Centre (n = 5 and n = 21), North (n = 3  
83 and n = 9), and South (n. 10 and n =17) (Fig S2).

84 3D models of skulls from the NHMUK were produced using a surface scanner (Creaform Go!SCAN  
85 20) at a resolution of 0.2 mm; 3D models of specimens from SMNH and NMS were generated with  
86 photogrammetry technique using Agisoft PhotoScan software (Agisoft PhotoScan,  
87 <http://www.agisoft.ru/>) [25]. Skulls were placed on a rotating plate and 36 photos were taken on each  
88 side (ventral, dorsal, lateral) using a Canon 30EOS camera. Pictures were scaled based on a scale  
89 using TpsDig and the Meshlab software [26,27]. Previous geometric morphometric studies  
90 demonstrated that skull size and shape variation in small and large mammals captured by 3D  
91 photogrammetry models is comparable to that obtained by 3D surface laser scanners, thus

92 generating a marginal measurement error compared to inter individual variation in landmarking data  
93 [28–31].

94 Each skull was landmarked three times by a single operator (LFR). The x, y, z coordinates of thirty  
95 anatomical landmarks were recorded using Stratovan Checkpoint (Stratovan Corporation [Davis,  
96 California, USA]) (original data are available in Supplementary Material S3). Following Russo et  
97 al. [22], landmarks were selected for their precision and representation of other cranial anatomy.

99

#### 100 *Morphometric analysis*

101 Landmark coordinates were subjected to Generalised Procrustes Analysis superimposition (GPA),  
102 using the function *gpagen* of the package “geomorph” [32], to remove the effect of size and spatial  
103 orientation [33]. Centroid Size (CS), defined as the square root of the sum of squared distances  
104 between each landmark and the barycentre of each configuration, was transformed into natural  
105 logarithm and used as proxy of skull size [34]. Skull shape variation between and within populations  
106 was explored using Principal Component Analysis (PCA) of the shape coordinates using the  
107 function *gm.prcomp* of the package “geomorph” [32,35] in the R environment [36]. We  
108 performed ANOVA on lnCS to assess the effect of different populations and sex,  
109 while Procrustes ANOVA was used to evaluate the effect of populations and sex  
110 on the variation in skull shape [37], using the function *procD.lm* of “geomorph”. Statistical significance  
111 for Procrustes ANOVA was obtained through randomized residual permutation (1000 runs) [38].

112 Directional (=DA) and fluctuating (=FA) asymmetry were measured for each population  
113 separately, using the *bilat.symmetry* function of “geomorph”. DA occurs when the average  
114 differences between left and right side are not significantly different from zero, while FA is the random  
115 deviations from perfect symmetry around a mean value of zero [39].

116 The Individual Signed Asymmetry Index, defined as the Procrustes distance between the right and  
117 left side [14,39], was quantified for each specimen. We used Kruskal Wallis test within each

118 population to test differences in asymmetry and size among time series. Mean shape of the FA  
 119 component was produced for each population to evaluate skull traits involved in FA.

120

## 121 **Results**

122 We found clear evidence of sexual dimorphism in both the size and shape of the skull of males and  
 123 females (Tab 1; Suppl. Mat. S4). We also found significant differences in size between the Swedish  
 124 and UK populations, the latter being larger than the former (Tab 1; Suppl. Mat. S4).

125 Also, PCA of the symmetric component of skull shape showed a clear separation in the morphospace  
 126 of the two populations (Figure 1). Otters from Sweden were characterised by a shorter and slender  
 127 snout and a narrower braincase compared to otters from UK. Procrustes ANOVA confirmed a  
 128 significant effect of population, sex and size on shape variation, although the two latter explaining a  
 129 smaller amount of shape variation (Tab1). No significant interaction was found among the three  
 130 factors, as evidenced in previous studies [22,23].

131

132 *Figure 1. Results from PCA run on the symmetric component of shape coordinates and wrapped 3d model*  
 133 *of extremes of variation along PC1.*

134

135

136

137 Tab 1. Top: results of Procrustes ANOVA testing the effect of population (UK vs Sweden), sex and size  
 138 (expressed by log centroid size) on skull shape variation. Bottom: results from ANOVA testing the effect of  
 139 population and sex on centroid size variation. Significance at  $p < 0.05$  are highlighted in bold.

<b>Procrustes ANOVA</b>							
	Df	SS	MS	Rsqr	F	Z	Pr.F.
Population	1.000	0.015	0.015	0.087	9.469	7.711	<b>0.001</b>
Sex	1.000	0.005	0.005	0.030	3.321	4.120	<b>0.001</b>
lnCS	1.000	0.004	0.004	0.026	2.847	3.913	<b>0.001</b>
Population:Sex	1.000	0.002	0.002	0.011	1.226	0.894	0.177
Population:Sex	1.000	0.002	0.002	0.012	1.318	1.128	0.135
Sex:lnCS	1.000	0.001	0.001	0.007	0.743	- 0.941	0.814
Population:Sex:lnCS	1.000	0.001	0.001	0.009	0.960	- 0.090	0.532

Residuals	89.000	0.138	0.002	0.817			
Total	96.000	0.169					
<b>ANOVA on lnCS</b>							
Population	1.000	0.044	0.044	0.067	9.347	2.381	<b>0.004</b>
Sex	1.000	0.172	0.172	0.264	36.777	4.665	<b>0.001</b>
Population:Sex	1.000	0.000	0.000	0.000	0.001	- 2.131	0.987
Residuals	93.000	0.435	0.005	0.668			
Total	96.000	0.650					

140138

141 The skulls of both populations were significantly affected by both directional (DA) and fluctuating  
 142 (FA) asymmetry, with the latter explaining greater variance (above 13%) in both populations (Tab 2).

143 Tab 2. Results of *bilat.symmetry* function, testing the effect of Directional Asymmetry (DA) and  
 144 Fluctuating Asymmetry (FA) on each population (UK and Sweden).

<b>Sweden</b>							
	Df	SS	MS	Rsq	F	Z	Pr..F.
ind	68.000	0.477	0.007	0.738	5.623	7.105	<b>0.001</b>
side (DA)	1.000	0.006	0.006	0.010	4.964	5.184	<b>0.001</b>
ind:side (FA)	68.000	0.085	0.001	0.131	4.400	22.435	<b>0.001</b>
error	276.000	0.078	0.000	0.121			
Total	413.000	0.647					
<b>UK</b>							
ind	28.000	0.219	0.008	0.696	4.418	3.122	<b>0.001</b>
side (DA)	1.000	0.005	0.005	0.016	2.809	3.845	<b>0.001</b>
ind:side (FA)	28.000	0.050	0.002	0.158	5.021	21.318	<b>0.001</b>
error	116.000	0.041	0.000	0.130			
Total	173.000	0.315					

145

146 Moreover, the two populations showed significant differences in Individual Signed Asymmetry Index,  
 147 with the highest value observed in the UK population (K-W chi-square = 8.1367, df =2, p = 0.004;  
 148 Figure 2).

149

150 *Figure 2. Box plots of median and SD of Individual Signed Asymmetry Index variation in Sweden*  
 151 *(yellow) and UK (red) (top), and in Sweden (red) and UK (yellow) populations before (< 1970), during*  
 150 *(1970-2000) and after (2001-2015) the decline (bottom).*

151

152 PCA run on the FA component showed a difference between the two populations only along PC2  
 153 (10% of cumulative variance) (Suppl. Mat. 5). Wireframes related to the mean FA component of  
 154 shape in the two populations evidenced the involvement of different skull regions (Fig. 3). In  
 155 Sweden FA emerged mainly in the zygomatic arch, whereas in UK it was concentrated in the  
 156 snout .

157

158

159 *Figure 3. Wireframes showing the regions of the skull involved in FA in the UK (left) and Swedish*  
 160 *(right) population.*

161

162 The Individual Signed Asymmetry Index differed significantly between the two time series only for  
 163 the UK population, with higher values during the decline time interval (1970 to 2000) (Tab. 3, Figure  
 164 2). We did not find any variation in size nor any correlation between size and Individual Signed  
 165 Asymmetry Index among the three time series (Tab. 3).

166 Tab. 3. Results of Kruskal Wallis test, testing the difference in Individual Signed Asymmetry Index  
 167 among the three periods (before, during and after the decline) in each population.

Signed.AI			
	Kruskal.Wallis.chi.squared	df	p.value
Sweden	0.453	2	0.797
UK	6.867	2	<b>0.032</b>
InCS			
Sweden	0.454	2	0.797
UK	5.260	2	0.0720

168

169

170

171

## 172 Discussion

173

174 Our results confirmed both sexual size dimorphism (SSD) and sexual shape dimorphism (SShD) as  
 175 possible adaptive strategies to reduce intraspecific competition in the Eurasian otter, similar to  
 176 evidences from other Mustelids [40–42]. We also observed significant differences in skull size and  
 177 shape between the Swedish and UK population. Specifically, UK otters exhibited a larger skull with  
 178 a shorter rostrum and a broader braincase compared to Swedish otters. Considering the  
 179 comparable latitudes of the two populations, this pattern likely arose from the island rule and, may



180 be attributed to dietary differences [43, 44]. In fact, Russo et al. [22] found differences in the shape  
181 of the skull in otters feeding on marine vs freshwater prey.

182 No significant differences were observed in skull size or shape among the three time intervals  
183 marking the otter decline in Europe in the last century. This is in contrast with findings by Yom-Tov  
184 et al. [45] who found an increase in body mass and zygomatic breadth in recent years in the otter  
185 population from Sweden.

186 In contrast, we revealed an increase in FA in both populations during the decline, with the UK  
187 population showing higher and significant FA values.

188 Similar results were found by Pertoldi et al. [46] in the Danish otter population, where an increase in  
189 FA was found in the early '70 of the last century and was attributed to pollution and habitat  
190 fragmentation. However, Pertoldi et al. [47] did not find any clear distinction in FA between healthy  
191 and endangered populations.

192 The Sweden and UK otter populations underwent a drastic decline in the last decades [2] likely  
193 exposing the populations to multiple stress factors that could have affected developmental processes  
194 and unusual levels of fluctuating asymmetry. One of the main causes of such declines was the loss  
195 and fragmentation of aquatic and riparian habitats, and the exposure to harmful pollutants such as  
196 polychlorinated biphenyls (PCBs) and organochlorine pesticides, that were banned in 1993 [2].  
197 Specifically, habitat fragmentation can reduce the chances of encounters between individuals,  
198 leading to bottlenecks and inbreeding depression [48,49]. Indeed, recent research has highlighted  
199 that the UK populations showed significant bottlenecks and recoveries between the 1950s and 1970s  
200 [50]. On the other side, as an apex predator, the otter can be greatly impacted by harmful pollutants  
201 that could interfere with ontogenetic processes and lead to developmental instability and increasing  
202 levels of FA [51–54]. In fact, a massive usage of PCBs in UK in the 1990s, and high levels of PCBs  
203 were found in otter carcasses collected during that period [55,56].

204 Stressed animals may also exhibit an increased susceptibility to parasitic infestations. Thus, the high  
205 rate of parasitism observed in otters from the UK may elucidate the high levels of fluctuating  
206 asymmetry (FA) detected in the UK otter population [57,58].

207 However, levels of PCB concentrations may vary from population to population, and individuals  
208 inhabiting the same areas may be affected by different levels of these compounds [59]. For example,  
209 in Sweden the population from the South showed higher concentrations of PCBs than the  
210 populations from the North [59]. Similarly, in the UK the population from Shetland showed higher  
211 concentrations of PCBs compared to the population from Wales [59]. Also, the exposure and  
212 concentration of PCBs in some individuals studied in the UK was probably higher than in those  
213 studied in Sweden. Finally, unlike the Swedish population, it should be underlined that UK otters  
214 experience no gene flow with other mainland European populations [60]. Moreover, although the UK  
215 population exhibits significant genetic variability among subpopulations [61,62], which has remained  
216 unchanged since the demographic population recovery, both in terms of heterozygosity and allelic  
217 diversity [63], the limited gene flow might have affected the capability to mitigate the effects of  
218 multiple stress during the population decline.

219 These evidences indicate individual populations in Sweden and UK have  
220 experienced diverse trends and pressures that might have resulted in different degrees of FA.  
221 Specifically, considering UK , the Shetland population is small and highly isolated, the Scottish  
222 population was a stronghold for otter survival in UK [62],  
223 whereas during the 1990s, there was a notable decline in otter populations from Wales and  
224 England [56]. Moreover, a recent population genomics insight [50] has highlighted the presence  
225 of introgression from Asian populations of *Lutra lutra* in East England, possibly related  
226 to imports of *L. l. barang* individuals from Asia into the UK in past decades. Similarly, in Sweden,  
227 southern populations have experienced a more significant decline compared to their northern  
228 counterparts, prompting the implementation of reintroduction programs in the South [64].  
229 Considering these multiple source of stress experienced by each subpopulation, additional  
230 sampling efforts are likely needed to disentangle the effect of each driver on FA and thus evaluate  
231 their potential impact on otters decline.

232

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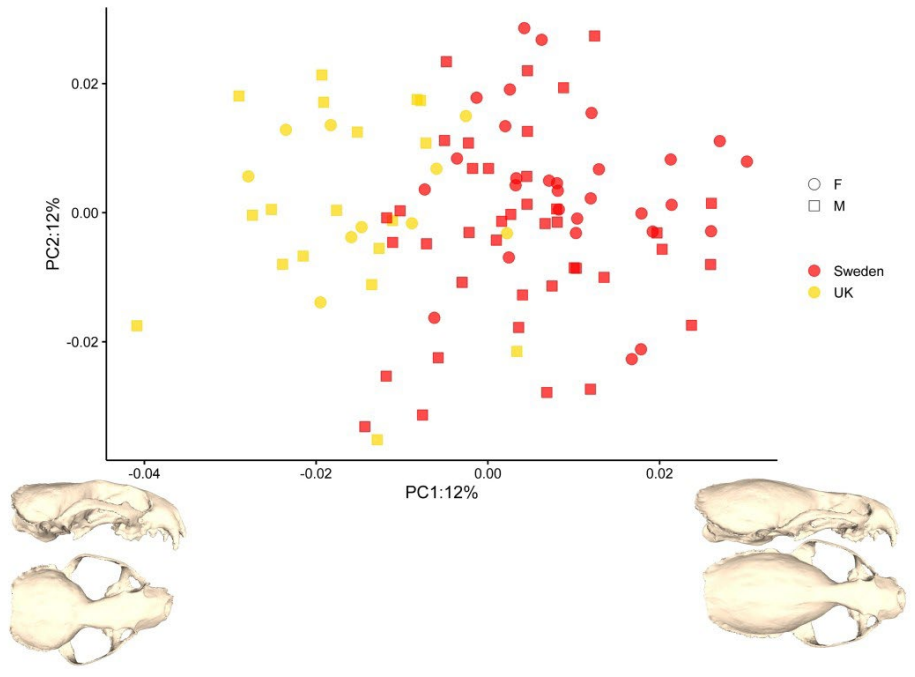


Figure 1. Results from PCA run on the symmetric component of shape coordinates and wrapped 3d model related to the extremes of variation along PC1

275x190mm (600 x 600 DPI)

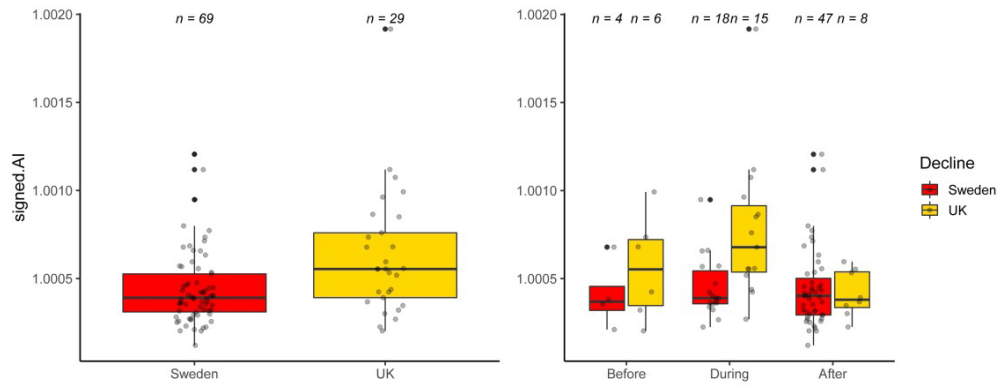


Figure 2. Box plots of median and SD of Individual Signed Asymmetry Index variation in Sweden (yellow) and UK (red) (top), and in Sweden (red) and UK (yellow) populations before (< 1970), during (1970-2000) and after (2001-2015) the decline (bottom).

203x81mm (600 x 600 DPI)



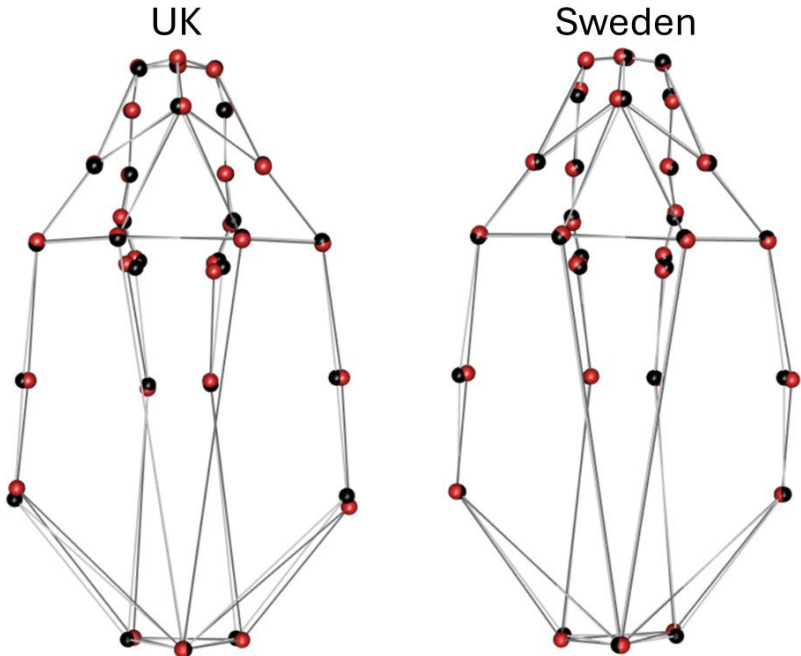


Figure 3. Wireframes showing the regions of the skull involved in FA in the UK (left) and Swedish (right) population.

275x190mm (600 x 600 DPI)