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Mandible shape in hybrid mice

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Abstract Hybridisation between closely related species is
frequently seen as retarding evolutionary divergence and
can also promote it by creating novel phenotypes due to
new genetic combinations and developmental interactions.
We therefore investigated how hybridisation affects the
shape of the mouse mandible, a well-known feature in evo–
devo studies. Parental groups corresponded to two strains of
the European mouse sub-species *Mus musculus domesticus*
and *Mus musculus musculus*. Parents and hybrids were bred
in controlled conditions. The mandibles of F_1 hybrids are
mostly intermediate between parental phenotypes as
expected for a complex multigenic character. Nevertheless,
a transgressive effect as well as an increased phenotypic
variance characterise the hybrids. This suggests that hybrid-
isation between the two subspecies could lead to a higher
phenotypic variance due to complex interactions among the
parental genomes including non-additive genetic effects.
The major direction of variance is conserved, however,
among hybrids and parent groups. Hybridisation may thus
play a role in the production of original transgressive
phenotypes occurring following pre-existing patterns of
variance.

Keywords Hybridisation · Mandible shape · Interspecific
divergence · Morphometrics

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Introduction

Diverging population may merge by coming into contact
before the full establishment of reproductive isolation, thus
forming hybrids and exchanging genes. As such, hybrid-
isation is often seen as a process swamping an ongoing
differentiation. The same process of hybridisation, however,
can also be seen as a source of genetic variation and
phenotypic novelty (Rieseberg et al. 1999, 2003; Bell and
Travis 2005). Indeed, if many hybrids appear to be
morphologically intermediate between both parental species
(e.g. Wilde and Echelle 1997; Auffray et al. 1996a; Loy et
al. 2001) or evidence an effect of dominance by being
closer to one of the parental groups (Thorpe and Leamy
1983), in numerous cases, they display original morphol-
ogies segregating from those of their parents (Monti et al.
2001; Valentin et al. 2002; Nolte and Sheets 2005). Such a
role of hybridisation as potential source of evolutionary
novelties is presumably the consequence of complex
genomic and epistatic interactions between the two diver-
gent genomes (Rieseberg et al. 2003; Seehausen 2004;
Albertson and Kocher 2005).

Among many phenotypic features, the mouse mandible
appears as a prominent model for evo–devo studies; the
more its genetics and development are dissected, the more
the complexity of the involved processes appears (e.g.
Klingenberg et al. 2001, 2003, 2004; Monteiro et al. 2005;
Zelditch et al. 2008). In the wild, the house mouse is also a

well-known biological model for the study of hybridisation, the most investigated case being the hybrid zone ranging through whole Europe between the two diverging subspecies *Mus musculus domesticus* and *Mus musculus musculus* (Boursot et al. 1993; Sage et al. 1993; Alibert et al. 1994). It thus provides a model to evaluate the impact of a hybridisation process that occurs in the wild on a complex phenotypic feature. Wild populations document how hybridisation occur among variable genomes but present several drawbacks: Environmental effects can participate to the divergence along a hybrid zone that may also correspond to a environmental gradient. Furthermore, the genealogy of the animals being unknown, effects of hybridisation cannot be dissected into effects related to successive generations of crosses; instead, wild hybrid populations have to be pooled (Valentin et al. 2002) or at best split into introgression classes (Auffray et al. 1996a). In contrast laboratory mice allow discarding most of the environmental variance that occurs in wild populations and controlling the pedigree of the mice considered. Considering inbred mice further discard most of the within-group genetic variance; therefore, phenotypic differences in hybrids can be interpreted as due to interactions among the two parental genomes instead as the product of a diffuse genetic variance. Hence, we focussed on hybridisation between two inbred laboratory strains established from wild-trapped animals and documenting the two European subspecies of house mice, *M. musculus domesticus* and *M. musculus musculus*, as well as their F_1 hybrids. The shape of the mandible was quantified in parents and hybrids using outline analysis of the mandibular bone, allowing the consideration of the patterns of divergence between groups. Such a characterisation of shape differences between parents and hybrids using the sophisticated methods of geometric morphometrics has been achieved in similar studies on other models (e.g. Monti et al. 2001; Valentin et al. 2002; Nolte and Sheets 2005), but we used the methodological potential of morphometric methods to go a step further. We used these methods to characterise directions of shape change related to allometry and the directions of greatest within-group variance. Such directions of greatest phenotypic variance have been suggested to be the expression of the underlying major genetic variance, thus constituting a potential channel to evolution (Schluter 1996) by producing most of the variance subsequently screened by selection. We thus investigated whether such directions were conserved among parents and hybrids and whether they played an evolutionary role in channelling the differentiation of the hybrids. Hybridisation may disrupt genomic co-adaptation and thus affect canalisation, i.e. processes allowing developmental pathways to buffer genetic differences within a population in order to produce homogeneous phenotypes among individuals despite genetic and environmental differ-

ences. By affecting such processes, hybridisation may also cause a disruption of the major direction of variance, a process that may be involved in the production of original phenotypes out of the range of the parent species. Furthermore, differentiated hybrid morphologies deviating from the expected intermediate morphology between parental groups are well known and referred as transgressive (Albertson and Kocher 2005). Transgressive hybrid morphology has been most often attributed to the new combination of alleles with antagonistic effects accumulated in the parental groups (Rieseberg et al. 1999, 2003); in such cases, a transgressive effect is expected from the F_2 generation onward. However, if non-additive genetic effects, such as regulatory networks and dominance, are involved, we might expect transgressive phenotypes from the F_1 before the occurrence of recombination between the two genomes. Finally, heterosis in overall size has been often documented in hybrids (Thorpe and Leamy 1983; Alibert et al. 1997). Increase in size may affect mandible shape due to allometric changes in the force required to move a larger, heavier mandible (Satoh 1997) or due to bone remodelling during a modified growth trajectory. Effects of hybridisation on size may thus contribute to the concomitant shape changes. We thus aimed to characterise hybrid morphologies within this rich conceptual background, with particular focus on the following questions:

- Are the F_1 hybrids at the mid-point in shape between parental groups, as expected for a complex trait influenced by many genes, or do they evidence non-additive effects such as dominance (hybrids being closer to one of the parental group) or transgression (hybrids deviating from the mid-line ranging between both parental groups)?
- Hybrids between European house mouse subspecies are known to often display a heterosis expressed as a size increase; can we characterise such an effect on size, and in this case, does allometric variation contribute to the occurrence of transgressive morphologies?
- Does hybridisation lead to an increased within-group variance and, possibly, to a destabilisation of the direction of greatest phenotypic variance?

Materials and methods

Material

The mice from the two parental groups correspond to two strains bred from wild-trapped animals and conserved at the Conservatoire de la souris sauvage (ISE-M, France). The western European house mouse *M. musculus domesticus* was represented by 33 mice from the strain WLA, derived

from mice caught near Toulouse (France) in 1976. The Eastern European subspecies *M. musculus musculus* was represented by 24 mice from the strain PWK, derived from mice trapped in Prague (Czech Republic) in 1982. The 38 F_1 hybrids were bred from these parental groups, from both WLA×PWK and PWK×WLA pairs. In order to buffer among-pair differences and to increase sample size, they were considered together. All the 95 animals were bred in the same controlled conditions at the ISE-M (Montpellier, France). Both the PWK and WLA strains have been established and maintained by brother/sister matings at the Institut Pasteur (Paris, France) to obtain inbred wild-derived mouse strains and had been maintained in these breeding conditions for more than 50 generations at the time of the experiment.

Methods

Outline analysis The shape of the mandible was estimated by its 2D outline, corresponding to the projection of the mandibular bone put flat on its lingual side. This outline provides a good description of the processes involved in the insertion of masticatory muscles as well as of the alveolar region hosting the cheek teeth and incisors. A radial Fourier transform (RFT) was applied to this mandible outline (Renaud and Michaux 2003), describing the initial data set as a sum of trigonometric functions of decreasing wave length, the harmonics, each weighted by two Fourier coefficients (FCs). The zero harmonic (A_0) is proportional to the size of each outline and is used to standardise all other FCs in order to separate size and shape components of form differences. Previous studies showed that considering the FCs of the first seven harmonics offered for the murine mandible a good compromise between measurement error, information content and number of variables (Renaud and Michaux 2003).

Excellent visual reconstructions, however, are provided by the alternative approach of the elliptic Fourier transform (EFT; Kuhl and Giardina 1982). This method presents, however, the drawback of inflating the number of variables by providing four FCs per harmonics. Both datasets are highly correlated, as shown by a Mantel test on distances between all specimens ($R=0.957$, $P_{observed\ correlation\ <\ random\ correlation} < 0.0001$). Therefore, the RFT was applied for statistical analyses and EFT only for providing reconstructed outlines.

Statistical analyses The size of the mandible was estimated by the zero harmonic of the RFT. Differences in size were tested using analysis of variance (ANOVA).

The shape of each outline was described by a set of 14 FCs (seven harmonics per two FCs). The pattern of shape differentiation was investigated using a principal compo-

nent analysis (PCA) on the pooled variance–covariance (VCV) matrix. Differences among groups were tested using multivariate ANOVA. The relationship of shape with size was investigated using a multivariate regression of the FCs on the size estimator A_0. To estimate the stability of the size–shape relationship across groups, a multivariate analysis of covariance (MANCOVA) was further performed, using groups as factor and size as covariate.

The patterns of shape differentiation obtained using different methods were compared using Mantel *t* test between distance matrices based on Euclidean distances among the specimens in the space of the FCs.

For each group, the major axis of variance was computed as the first eigenvector of the VCV matrix based on the 14 FCs per mandible. Directions of allometric variation corresponded to the slope of the regression line of the multivariate regression of the FCs on A_0. The angle between such directions of shape change corresponds to the arc cosine of the inner product of the two vector elements. The distribution of angles between simulated random vectors was used to assess the statistical significance of this correlation (Klingenberg 1996; Renaud et al. 2006). Fifty thousand simulations provided the following significance threshold for the probability that $R_{observed} > R_{random}$: $P>0.95$, $R=0.517$; $P>0.99$, $R=0.651$; $P>0.999$, $R=0.770$.

The sampling of the initial groups may affect the evaluation of the shape parameters. The robustness of the direction of morphological change was thus investigated using a bootstrap procedure of the initial groups. Each group was bootstrapped 1,000 times, and allometric directions and major axes of variance were estimated on these bootstrapped samples. The distribution of these bootstrapped directions around the original one was described by providing the mean and median coefficients of correlation and angles, as well as their values including 75% and 95% of the correlations (Renaud and Auffray 2009).

Results

Mandibles of both parental groups are of similar size ($P=0.689$; Fig. 1) but differ in shape ($P<0.001$). F_1 hybrids display a heterotic effect on size, being larger than both WLA and PWK mice ($P<0.001$). They also differ in shape from both parental groups ($P<0.001$). On the first principal plane of a PCA (Fig. 2), F_1 hybrids appear overall intermediate between both parental groups along the first axis, representing 55% of the total variance. They exceed parental values along the PC2 (28% of variance), however, evidencing the existence of transgressive phenotypes. Shape differences are distributed all over the mandible (Fig. 2b). PWK mice are characterised by a reduced,
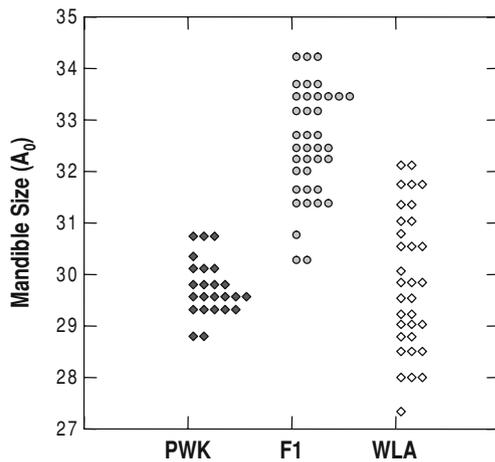
Fig. 1 Mandible size in parental groups (PWK, *Mus musculus musculus* and WLA, *M. musculus domesticus*) and their F_1 hybrids. Each dot corresponds to an individual measure. Size is estimated by the zero harmonic of the Fourier outline analysis

straight coronoid process, a long condyle, and a thin alveolar region, whereas WLA mice show a well-developed, arched coronoid region, a short condyle and a massive alveolar region. Intermediate and yet transgressive shape in hybrids is exemplified by the coronoid region, sharing a straight shape with PWK parents but its overall length with WLA mice.

F_1 hybrids not only exceed parental values but also display the highest total variance, estimated as the trace (=sum of diagonal elements) of the VCV matrix of the FCs (Fig. 3).

Because the heterotic effect on size may be suspected to participate to the transgressive effect on shape due to allometric shape changes, allometric variations were investigated within and among groups. A MANCOVA evidenced effects of group, size and interaction in the shape differentiation (group, $P=0.004$; size, $P=0.001$; interaction, $P=0.005$), but allometry appears as a minor source of shape variation since a multivariate regression of the FCs on size is only significant in the WLA group (WLA $P<0.001$) showing the largest size variance (Fig. 1). Regressions are significant in neither PWK nor F_1 groups (PWK, $P=0.229$; F_1, $P=0.062$). In agreement, directions of allometry are poorly estimated in the PWK and F_1 groups, a stronger estimate being achieved in the WLA group (Table 1). These directions of within-group allometry are further discrepant among groups (Table 2; Fig. 2) as well as discrepant with the allometric relationship calculated on all specimens ($P<0.001$). Yet, to disentangle the intra-group "static" allometry from the intergroup "evolutionary" allometry (sensu Klingenberg 1996), residuals from the size–shape regression within each group were considered as new, "size-free" shape parameters. Discarding the intra-group allometry does not change much the relationships among specimens (Mantel tests between distances calculated among individuals based on raw FCs and residuals: WLA, $R=0.919$; PWK, $R=0.946$; F_1, $R=0.987$; in all cases $P_{\text{observed correlation}<\text{random correlation}}<0.0001$). Adding the group mean to these residuals provided a new complete dataset, where static intra-group allometry was discarded. The emerging pattern of shape differentiation is highly correlated on the one provided by the raw FCs

Fig. 2 Mandible shape variation between parental groups (PWK, *Mus musculus musculus* and WLA, *M. musculus domesticus*) and their F_1 hybrids, visualised on the principal plane of a PCA on the variance–covariance matrix of the FCs of the outline analysis. Major directions of within-group variance (V1 of the VCV matrix; *full arrows*) and of within-group and evolutionary allometry (*dotted arrows*) have been projected unto this first principal plane. Reconstructed outline, obtained using the EFT and visualising the average shape of the WLA, PWK and F_1 groups are displayed on the *lower panel*

Size Variance
A₀ coefficient off variation

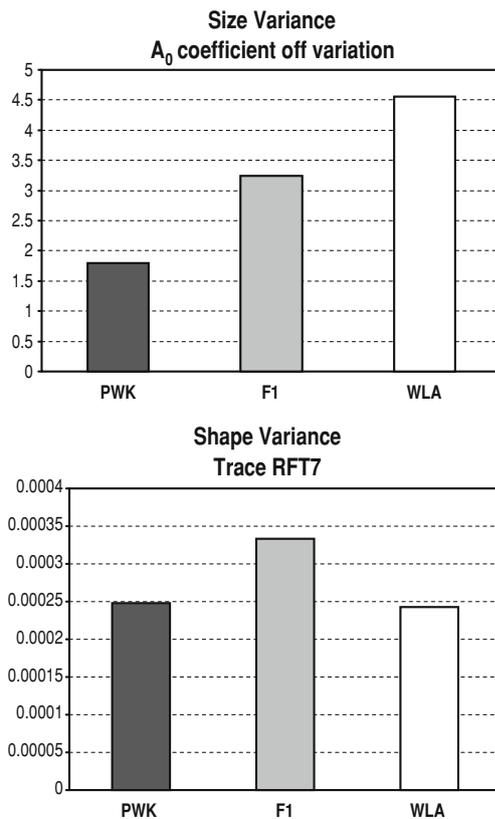

Shape Variance
Trace RFT7
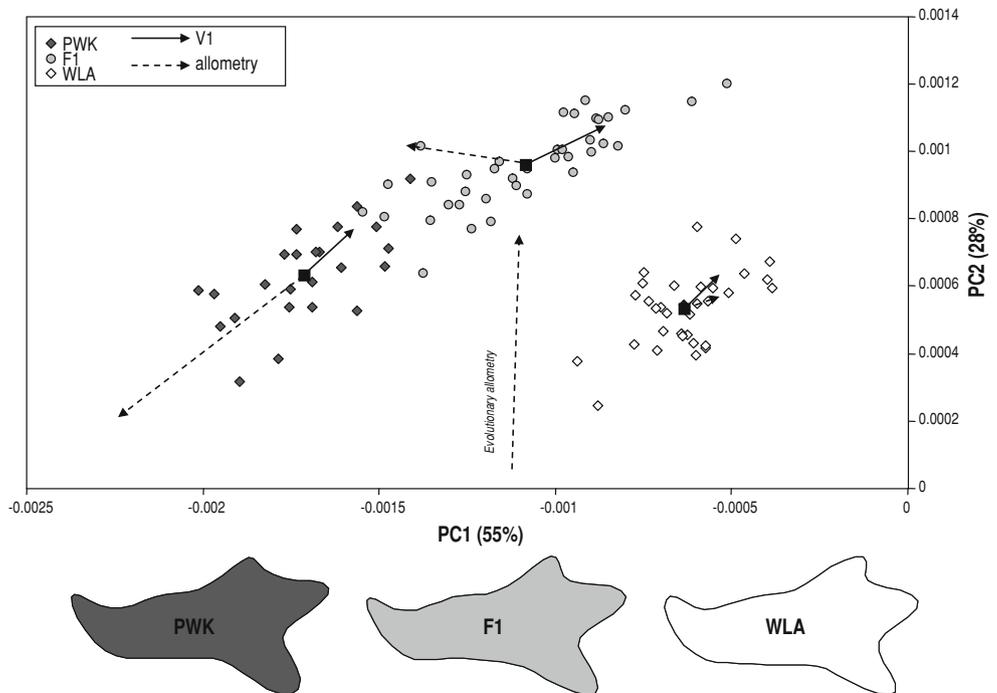
Fig. 3 Size and shape variance in the parental groups (PWK, *Mus musculus musculus* and WLA, *M. musculus domesticus*) and their F_1 hybrids. **a** Size variance, estimated as the coefficient of variation ($=100 \times \sqrt{\text{variance}}/\text{mean value}$) of the zero harmonic of the Fourier outline analysis. **b** Shape variance, estimated as the trace of the variance–covariance matrix ($=\text{sum of the variance of each variable}$) of the Fourier coefficients of the outline analysis

(Mantel $R=0.989$, $P_{\text{observed correlation}<\text{random correlation}} < 0.0001$).

Major directions of variance within each group are robustly estimated (Table 1), especially for PWK and F_1 hybrids. The major direction in the WLA group is less

robustly estimated, with the occurrence of permutation with the second eigenvector, correlated with allometric variation ($R=-0.779$), which is the most pronounced in this group probably due to its large variance in size. In comparison with allometric directions and directions of greatest variance, directions characterising the shape differences between groups appear as very robustly estimated (Table 1).

V1 vectors are all correlated but particularly between PWK and F_1 hybrids (Table 2). The shape change from PWK to F_1 hybrids follows this major axis of intra-group variance (Table 2), as shown by the parallel and aligned distributions of PWK and F_1 hybrids in the PCA shape space (Fig. 2).

Discussion

The first result emerging from this study is a clear heterotic effect on mandible size in F_1 hybrids of laboratory animals representing the European subspecies of house mice *M. musculus domesticus* and *M. musculus musculus*. This is fully congruent with previous work showing a similar heterotic size increase on wild hybrids (Alibert et al. 1997). A further aspect of heterosis has been evidenced on *M. musculus domesticus* × *M. musculus musculus* hybrids regarding developmental stability, i.e. the ability of individual to buffer perturbations occurring during the development of morphological structures. Lower fluctuating asymmetry is interpreted as a marker of a better developmental stability and has been evidenced in wild (Alibert et al. 1994) as well as in laboratory hybrids (Auffray et al. 1996b; Alibert et al. 1997). All these results point to a hybrid vigour despite indications of disruption of some gene complexes revealed by dysfunction in the immune response to some parasites (Moulia et al. 1991) and incompatibility between genetic markers located on sexual chromosomes (Dod et al. 1993).

A further aspect of our results is an increased variance of mandible shape in hybrids. This high variation of F_1

Table 1 Distribution of the correlation ($R=$inner product) and angle (arccosine of R) between original directions of change and 1,000 bootstrapped estimations

	Mean		Median		75%		95%	
	R	Angle	R	Angle	R	Angle	R	Angle
WLA–PWK	0.997	4.0°	0.998	3.8°	0.997	4.8°	0.993	6.9°
WLA–F₁	0.994	5.7°	0.996	5.4°	0.993	7.0°	0.985	9.9°
PWK–F₁	0.996	4.7°	0.997	4.5°	0.995	5.7°	0.991	7.7°
WLA allometry	0.874	26.8°	0.915	23.8°	0.834	33.5°	0.625	51.3°
PWK allometry	0.726	39.8°	0.810	35.9°	0.630	50.9°	0.171	80.1°
F₁ allometry	0.787	35.8°	0.824	34.5°	0.703	45.3°	0.476	61.6°
WLA V1	0.806	30.9°	0.915	23.7°	0.729	43.2°	0.255	75.2°
PWK V1	0.962	14.1°	0.977	12.4°	0.955	17.2°	0.886	27.6°
F₁ V1	0.990	7.4°	0.993	6.7°	0.988	8.7°	0.971	13.8°
Total V1	0.989	7.8°	0.993	6.8°	0.985	10.0°	0.962	15.9°

Mean and median of the distribution are provided as well as values including 75% and 95% of the distribution of the thousand correlations. *V1* First eigenvector of the VCV matrix of the FCs. *Total V1* First axis of the PCA on all specimens

Table 2 Correlations among vectors of shape changes, directions of within-group allometry and major directions of within-group variance (*V1* first eigenvector of the VCV matrix of the FCs)

	WLA–PWK	WLA–F$_1$	PWK–F$_1$	WLA allom	PWK allom	F$_1$ allom	WLA V1	PWK V1	F$_1$ V1
WLA–PWK	–								
WLA–F$_1$	0.685	–							
PWK–F$_1$	−0.721	0.010	–						
WLA allometry	−0.121	−0.097	0.074	–					
PWK allometry	0.403	−0.191	−0.736		–				
F$_1$ allometry	0.667	0.419	−0.517			–			
WLA V1	−0.361	0.212	0.698	0.175	−0.528	0.021	–		
PWK V1	−0.430	0.228	0.808	0.416	−0.340	−0.061	0.779	–	
F$_1$ V1	−0.616	−0.063	0.808	0.441	−0.222	−0.187	0.731	0.900	–
Total V1	−0.989	−0.595	0.791	0.177	−0.419	−0.625	0.470	0.554	0.724

For each comparison between pair of vectors, *R* (inner product of both vectors) is given. *R* values in italics corresponding to $P_{R\ observed > R\ random} >$ 0.99 ($R > 0.651$). *Total V1* First axis of the PCA on all specimens

hybrids between inbred mice cannot be attributed to an increased genetic variance, contrary to what may happen in natural hybrid zone because F$_1$ hybrids between inbred strains all share the same pool of alleles. Non-additive genetic effects may participate to this increased variance, including sex-linked effects that were not presently addressed due to the limited sample. Hybrids may be further characterised by a decreased canalisation that is generally defined as processes allowing developmental pathways to buffer genetic differences in order to produce homogeneous phenotypes among individuals despite genetic and environmental differences.

Discrepancies between patterns of increased variance, evidenced by our analysis, and higher developmental stability, evidenced in previous studies on other hybrid mice (Alibert et al. 1994, 1997), may appear surprising in the first place but may be the rule rather than an exception (Debat et al. 2000; Debat and David 2001; Rego et al. 2006) because the two signals are relevant of different processes. Original genomic combinations in hybrids, due to the coexistence of alleles originating from two divergent genomes, may lead to the production of a higher phenotypic variation within a population, notwithstanding that each individual specimen might benefit of a good developmental stability.

This result raised the question of the role of hybridisation in producing new, original phenotypes. Previous studies on skull shape of wild hybrids through the Danish hybrid zone between the two subspecies evidenced a smooth shape gradient from one parental form to the other, paralleling the gradient of genetic introgression of one subspecies into the other (Auffray et al. 1996a). Given the complex genetic architecture of the mouse mandible, involving numerous genes with partly redundant effects (Klingenberg et al. 2001, 2004), one might expect that the effects of the numerous genes interacting and compensating

each other would achieve an intermediate shape in hybrids. This is indeed the case considering the first-order signal characterising our F$_1$ hybrids. Few dominance effects are evidenced, since hybrids are close to the mid-point between parental groups rather than close to one of the parental strain. The present F$_1$ hybrids further display a transgressive aspect of their phenotype, exceeding the parental range of variation as a second-order signal (PC2 on Fig. 2) in the pattern of phenotypic differentiation. Transgressive hybrids have been argued to be unexpectedly common (Rieseberg et al. 1999; 2003). The ordinary interpretation of transgressive phenotypes, however, involves the occurrence of recombination creating original genetic association from the F$_2$ generation onwards (Albertson and Kocher 2005; Albertson et al. 2005; Bell and Travis 2005). Our results may document a further case of transgressive hybrids in animals, since this effect occurs already in the F$_1$ generation where recombination did not occur yet. It suggests that genomic interactions between the two genomes can already occur and produce original phenotypes by producing new genomic conditions, regulatory networks and/or association of genes.

It has been argued that features under constant directional selection would be less prone to accumulate genes with antagonistic effects, contrary to features either diverging under stabilising selection, drift or fluctuating directional selection (Albertson et al. 2003; Albertson and Kocher 2005). Accordingly, transgressive hybrids seem to be more frequent in intra-specific than inter-specific crosses, the former supposed to occur rather by drift whereas ecological shifts among species suggest the more frequent occurrence of directional selection in inter-specific divergence (Rieseberg et al. 2003). The case of the two subspecies of mice would be intermediate in that respect. The mandible of rodents indeed seems to have diverged

among species as an adaptive response to the feeding aspects of ecology (Michaux et al. 2007), but within groups sharing a similar diet, shape differentiation seems to have occurred according to drift (Renaud et al. 2007). The two subspecies of mice share very similar ecological characteristics, and the divergence of their mandible shape most probably occurred by drift during their independent history since divergence, ~500,000 years ago (Boursot et al. 1993). Further divergence due to drift might have further occurred within each inbred strains since their establishment in the 1980s. In that respect, the occurrence of transgressive phenotypes follows the expectation for features that have not been under constant directional selection.

An alternative, not mutually exclusive, explanation for transgressive phenotypes in these hybrid mice involves allometric shape variations. As formerly mentioned, the hybrids between the two subspecies have been shown to display hybrid vigour that translates into not only better developmental stability but also a larger mandible size. Since larger, heavier mandibles requires relatively more force to achieve masticatory movements (Satoh 1997), allometric relationship between mandible size and shape can be expected and indeed have been evidenced at a larger taxonomic scale within the murine family (Michaux et al. 2007). Within the house mouse, insular divergence is occasionally associated with larger size increase according to the "island rule" for small mammals (Lomolino 1985, 2005), and accordingly, insular divergence of the mouse mandible has been found to match directions of allometric variation in some cases (Renaud and Auffray 2009). Allometry may thus be a tempting explanation for the transgressive phenotype of mandibles also characterised by a large size. The explanatory role of allometry in the hybrid divergence would have been convincingly demonstrated if within-group "static" allometry in the parental groups were parallel in direction and served as guide to the evolutionary allometry characterising the divergence of the larger mandibles of the hybrids. We failed, however, to evidence such consistent within-group allometric directions. Allometry was neither significant within PWK nor hybrid groups. The estimate of allometric direction is indeed very sensitive to sample size (Cardini and Elton 2007), and the sampling of our groups may be at the limit of an acceptable sample. Another difficulty is that we consider animals bred in laboratory conditions from strains with a lowered genetic diversity compared to wild populations. The expected reduction of size and shape variances in these samples may limit the assessment of the relationships between both variables. In contrast, the estimate of major axes of variance has also been advocated to be sensitive to sample size and to be only robust in large samples ≥30 specimens (Polly 2005), but such directions of greatest variance appeared to be robustly estimated and conserved in all groups including

the hybrids. Hence, the increased variance in hybrids did not lead to the production of phenotypes according to another direction or to a fuzzy within-group structure leading to unclear patterns of major variance. On the contrary, it shows that the production of original transgressive phenotypes occurred following pre-existing patterns of variance. These directions were particularly well conserved between the *M. musculus musculus* group and the F_1 hybrids, and the differentiation of from this parental group to the hybrid also paralleled this common direction of greatest variance. The pattern of within-group variance of *M. musculus musculus* seems thus to have been dominant over the one of *M. musculus domesticus* and possibly to have served as channel to the divergence of the F_1 hybrids. This may corroborate the role of these major directions of within-group variance as channels to phenotypic evolution (Schluter 1996).

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