**Insect head modularity allows conspicuously asymmetric but functionally constrained mandible shapes**

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**Introduction**

Bilateral symmetry is one of the most widespread morphological characters in animals. Yet, even animals with a well-defined bilaterally symmetrical body plan (e.g. most Bilaterians) are generally *not strictly* symmetrical, since many structures such as vertebrate internal organs are obviously asymmetrical or even unilateral (Babcock, 2005; Palmer, 1996; Van Valen, 1962). The breaking of symmetry can however take many other forms, sometimes less evident, with different biological implications (Klingenberg, 2022; Palmer, 1994; Van Valen, 1962).

The differences between left and right sides can be subtle, requiring precise quantitative measurements to be detected, or conspicuous, i.e. visible directly upon observation. Among subtle asymmetry types, fluctuating asymmetry is arguably the most studied, due to being an assumed proxy for developmental instability, relating to intrinsic or extrinsic stresses on organisms (Benítez et al., 2020; Graham et al., 1993; Klingenberg, 2022; Møller, 1990; Palmer, 1994; Savriama et al., 2016; Van Valen, 1962). When studying fluctuating asymmetry, it is assumed that symmetry is the norm and the target phenotype to achieve (Hansen et al., 2006), and that stresses will lead to small random left-right differences, the distribution of which should be normal and centered on 0 (i.e. on perfect symmetry) at the population level (Palmer, 1994). Subtle asymmetry types also include directional asymmetry and antisymmetry, in which a symmetrical phenotype is *not* the norm, with the left-right differences being of consistent direction in the case of directional asymmetry, leading to a normal distribution *not* centered on 0, or of random direction in antisymmetry, leading to a platykurtic or bimodal distribution of differences. Directional asymmetry and antisymmetry are also found, and generally characteristic for conspicuous asymmetries (Palmer, 2004). In this case again, a symmetric phenotype is not the norm, and the target phenotype is visibly different between left and right sides.

Because directional asymmetry and antisymmetry entail systematic differences between sides, they are generally considered to be inherited, and possibly to be adaptive. The latter point is however dependent on whether subtle or conspicuous asymmetries are studied. For example, *subtle* directional asymmetries in insect wings have been repeatedly demonstrated, but may be of little adaptive significance, due to limited functional consequences (Klingenberg et al., 1998; Pélabon & Hansen, 2008; Pither & Taylor, 2000). On the other hand, *conspicuous* directional asymmetry in the Cetacean skull has been argued to be related to feeding or biosonar function (Churchill et al., 2019; del Castillo et al., 2016; Huggenberger et al., 2017; Lanzetti, 2022; Macleod et al., 2007); conspicuous directional asymmetry in the skulls of flatfishes relates to their benthic ecology (Evans et al., 2021); conspicuous antisymmetry in many Crustacean appendages has been related to divergent left and right functions (Govind, 1989; Govind & Blundon, 1985; Levinton, 2016; Pratt & Mclain, 2002); and in humans and mice, the mirroring of internal organs, or *situs inversus*, is mostly problematic when only some of the organs are concerned, while complete *situs inversus* entails few health defects (Palmer, 2004).

If one considers that there is not just one left-right (or right-left) body axis, but instead two opposite medio-lateral axes (Meinhardt, 2001; Palmer, 2004), the emergence of conspicuously different left and right phenotypes can be interpreted as the relatively independent expression of two different developmental programs, which can be initiated genetically (e.g. usual *situs solitus*), randomly (e.g. *situs inversus* in *iv* mutant mice, (Brown & Wolpert, 1990)), or environmentally (e.g. lobster claws (Govind, 1989)). It should however be kept in mind that even in extreme cases of antisymmetry (e.g. in fiddler crabs), the development of both sides has been shown to be positively related, at least in terms of resource investment (Levinton, 2016). With this view in mind, in cases of conspicuous directional asymmetry or antisymmetry both medio-lateral axes may be akin to *quasi-autonomous components* (Wagner et al., 2007; Zelditch & Goswami, 2021), producing a special case of modularity in which left and right sides represent developmental modules. In addition to being developmentally modular, each side may also represent a quasi-autonomous functional module, perhaps most obviously in male fiddler crabs which use their minor claw to feed and their major claw for fights and displays, certainly leading to very different selective pressures on the opposite sides of the animal (Pratt & Mclain, 2002). Although the modularity of crab claws has to our knowledge not directly been assessed, studies on the Cetacean skull do suggest a link between directional asymmetry and a modification of the modularity pattern of the skull (Churchill et al., 2019; del Castillo et al., 2016, 2017). The situation in this latter case is slightly more entangled than in the crab claws example, because both sides of the skull, which may develop differently, may be involved in the same function, here feeding or sound production. On the other hand, the general shape of the skull should achieve some degree of symmetry to retain hydrodynamic properties, which would require that some skull modules develop symmetrically, while others develop asymmetrically.

Another case of conspicuous asymmetry, are the mandibles of many insects, among which many Orthopterans, i.e. grasshoppers, crickets and relatives (Ball, 1992; Chapman, 1964; Clissold, 2007). Mandibles are generally used to shear and crush plant material, and their asymmetric shapes allow the distal parts (incisor) to cross and act as double blades, and the proximal parts (molar) to occlude (Clissold, 2007). The left and right mandibles each rotate around an axis formed by two articulation points with the head. They can move independently from each other and, in Orthopterans, are each actuated by one adductor and one abductor muscle which are attached to the inside of the head capsule cuticle (Clissold, 2007). Contrary to the bones of the Cetacean skull, the Orthopteran mandibles remain to some extant physically independent from each other, while contrary to the fiddler crab claws, both mandibles must work together to achieve their feeding function. The Orthopteran head therefore constitutes a somehow intermediate study case of an integrated structure, or *tagma* (Minelli et al., 2013), combining symmetric and asymmetric components, with the left and right asymmetric components being to some extent physically independent, but sharing a common function. In addition to a common function, left and right mandibles share a common developmental origin, being derived from a single of the highly modified head segments (Posnien & Bucher, 2010).

We propose that (i) to achieve conspicuous asymmetry, by which we mean visibly different morphologies on the right and left sides of an animal, the opposite medio-lateral axes of development must have some degree of autonomy from each other, possibly forming modules. At the same time, because overall body or tagma symmetry should be generally maintained, we may expect that (ii) asymmetric structures should be to some degree autonomous from symmetric ones, possibly *requiring* some pre-existing level of modularity to allow for asymmetry to arise evolutionarily. (iii) In the case where the left and right asymmetric components must work together to achieve a given function (like the Orthopterans mandibles) and/or when they are physically tightly linked (as in the Cetacean skull), the functional or architectural constraints may also counteract the potential right-left developmental modularity suggested in point (i). Another possibility is that (iv) asymmetries may “spill-over” from conspicuously asymmetrical modules to neighboring areas, for example through asymmetric mechanical loads producing plastic development or reinforcement preferentially on one side or through correlated growth (Levinton, 2016; Pratt & Mclain, 2002; Tiwari et al., 2017).

To test these hypotheses, we focus here on the Orthopteran head morphology, which we quantify using geometric morphometrics. We measure morphological variation in adults at the population level, and decompose it into various types of asymmetries. Taking advantage of the multivariate nature of geometric morphometric data, we study covariation patterns between traits to test different variational modular partitions (Klingenberg et al., 2001; Wagner et al., 2007; Zelditch & Goswami, 2021) and compare them to each other. We expect that mandibles should show strong directional asymmetry, as shown qualitatively in the literature, and that their asymmetrical mechanical actions may induce asymmetry in the neighboring regions, but less so in more distant regions of the head, especially in sensory structures for which symmetry may be functionally advantageous (e.g. compound eyes). Second, we expect that, according to point (i) in the previous paragraph, the left and right mandible may constitute separate modules, allowing their divergent morphologies to emerge, and that they are also quasi-autonomous with regard to the head capsule structures, in which symmetry should be maintained (point (ii)). Alternatively, because the mandibles should enable proper shearing and occlusion, it may be expected as suggested by point (iii), that they are tightly integrated, forming one functional and variational module. If this is the case, it may also be expected that the level of asymmetry is tightly controlled, showing strong adaptive accuracy (Hansen et al., 2006; Pélabon & Hansen, 2008), with individuals deviating from the optimal level of asymmetry having worse biting performance.

**Materials and Methods**

*Specimens and measurements*. Forty-nine live adult specimens of *Schistocerca gregaria* were purchased from Fressnapf© (Krefeld, Germany). The animals were brought back to the lab, and their maximal bite forces were measured using the setup developed by (Rühr & Blanke, 2022) on the same day or the next. Bite forces were measured by two different users (SG and SS), by holding the animals between thumb and index, and allowing them to bite voluntarily on the bite plates. Continuous sequences of bites were recorded, and the highest measured bite force (i.e. maximal voluntary bite force) was extracted and used in further analyses. The sex of individuals was determined, and five linear measurements were obtained: body length (BL), pronotum width (PW), head width (HW), head length (HL) and head height (HH).

*Fixation and microCT scanning*. Specimens were then fixed in Bouin solution for around 3 days, their heads were cut off the body, and rinsed repeatedly in 70% ethanol. Following this, the heads were dehydrated in a series of increasingly concentrated ethanol solution, going from 70% to 100% by steps of 10%, with 1 hour at each step. After this process, the heads were critical-point dried (Autosamdri 931.GL) before microCT scanning using a Bruker SkyScan 1272 (voltage = 50 kV, current = 200 µA, Image pixel size = 6.0 µm or 7.5 µm) and reconstructed using NRecon. All heads had fully closed mandibles when scanned.

*Landmarking*. 3D reconstructed heads were imported and rendered in the software MorphoDig (Lebrun, 2018). In total, 38 homologous landmarks were placed across the head. However, three landmarks had to be excluded because they could not be placed accurately in all individuals, leaving a total of 35 landmarks (Fig. 1). All landmarks were digitized by the same user (SG) and replicated once, to allow discrimination between the various components of inter- and intra-individual shape variation (i.e. asymmetry, see below).

*Shape variation decomposition and analysis of asymmetry*. Entire landmark configurations were used, meaning we considered here the object symmetry of the head and mandibles as a whole. Individual shapes and their replicates were aligned by partial Generalized Procrustes Analysis, using functions from (Claude, 2008). Two different approaches were used in the decomposition of asymmetric variation. First, we implemented the approach from (Neubauer et al., 2020), with custom code, to obtain estimates of fluctuating asymmetry (FA) and directional asymmetry (DA) at the individual level. This relies on the use of non-centered PCA on the matrix of differences between the configuration of a given individual and its reflection across the sagittal plane. In the resulting PCA, the center (coordinates [0, 0]) has a biological meaning, since it represents perfect symmetry (i.e. no difference between a configuration and its reflection). If the population average coordinate along one of the PC axes is significantly different from 0, it can be concluded that this axis represents DA variation. Individual positions (averaged across replicates) along this axis can therefore be used as a proxy for “individual directional asymmetry” (iDA) in shape differences represented by this axis. When the population average along an axis is not different from 0 (and the distribution is not bimodal, which would suggest antisymmetry), the individual positions can serve as a proxy for “individual fluctuating asymmetry” (iFA). In addition, individual total asymmetry (iTA) was also computed as the distance between a configuration and its reflection, averaged across replicates for the same individual. The second decomposition approach estimates DA and FA (as well as inter-individual variation) as population-level values, and relies on ANOVA (Palmer, 1994), as implemented in the geomorph R package (function bilat.symmetry) (Adams & Otárola-Castillo, 2013). The ANOVA has two explanatory factors, individual (representing inter-individual variance), side (or mirroring, representing DA), with their interaction representing FA. Finally, using replicates allows one to take into account the landmarking error, and to test for significance of DA and FA.

*Modularity and integration analyses*. Replicated landmark configurations for each individual were averaged, and the resulting shapes were used in the following analyses. In this study, we test strictly only variational modularity (i.e. whether the grasshopper head shows stronger covariation within than between regions) (Zelditch & Goswami, 2021). We did not use exploratory but confirmatory approaches to test for modularity. This entails defining *a priori* different modular partitions to be tested against the null hypothesis of no modularity, and tested against each other. The landmarks were therefore sorted into six different partitions (Fig. 2): (i) a 2 modules “Head-Mandibles” partition, in which all landmarks placed on both left and right mandibles were gathered into one module, while all other landmarks, placed on the head capsule and sensory structures, formed the second module. (ii) a 3 modules “Head-Mandible-Sensory” partition, in which mandible landmarks form one module, landmarks from sensory structures (eyes, antennae, ocelli), which are in the dorsal half of the head form the second module, and landmarks from the ventral half of the head capsule and frons/clypeus form the third module. (iii) a 3 modules “Head-Mandibles asymmetric” partition in which landmarks from each mandible (left and right) form two separate modules, while the rest (entire head) constitute the third module. (iv) a 4 modules “Head-Mandible asymmetric-Sensory” partition, in which left and right mandible constitute separate modules, and head landmarks are split between the dorsal (i.e. sensory) and ventral regions. (v) a 2 modules “Ventral-Dorsal” partition, in which landmarks from both mandibles and the ventral part of the head form a single module, while the dorsal head landmarks constitute the second module. (vi) a 2 modules “Half-Half” partition, in which the left half and right half of the head constitute separate modules, with midline landmarks excluded from the analysis. These different partitions were tested against the null hypothesis of no modularity, and ranked against each other using two of the most widely used current approaches: EMMLi (Evaluating modularity with maximum likelihood), implemented in the EMMLi R package (Goswami & Finarelli, 2016), and CR (Covariance ratio), implemented in the geomorph R package (Adams, 2016; Adams & Collyer, 2016). Because EMMLi has been shown to have high false positive rates and to strongly favor models with more parameters (Adams & Collyer, 2019), we only show these results in details in the Supplementary Material. Integration between modules was also tested pairwise for each partition using two-block partial list squares (PLS), as implemented in geomorph. Because discussions are ongoing about the impact of Procrustes superimposition on results of modularity analyses (Cardini, 2019, 2023; Zelditch & Swiderski, 2023), we ran our modularity tests and comparisons a second time, while applying a second "local" superimposition module by module. In other terms, the globally superimposed coordinates array was split into "module by module" arrays, which were individually superimposed again. These "module by module" coordinate arrays were then concatenated back together in an array of the same dimensions as the original one, and with landmarks in the same order, before running modularity tests and comparisons once more. Although this process entirely removes the biological spatial relationship between modules, it does not preclude analyses of covariances. We then compare results from both approaches (i.e. global superimposition and module by module superimposition).

*Variability and correlations between bite force and asymmetry.* To test whether the degree of asymmetry in the head and mandibles was functionally constrained, we assessed the correlation between individual *in vivo* maximum voluntary bite force (BF), and the various indices of individual asymmetry (iTA, iFA, iDA). One hypothesis was that a functional “key and lock” principle for good occlusion between mandibles would lead to an optimum asymmetry value maximizing bite forces, and therefore to a quadratic relationship between BF and iTA or iDA. On the other hand, FA is generally considered to relate to worse fitness, therefore possibly to a negative relationship between iFA and BF. Finally, to test whether these traits may be under selection, we computed their respective coefficients of phenotypic variation (CVp), which can be viewed as a measure of adaptive accuracy (Hansen et al., 2006; Pélabon & Hansen, 2008).

**Results**

*Head shape asymmetry*. As expected, head shape at the population level was strongly directionally asymmetric (Table 1, Fig. 3). This directional asymmetry (DA) is however located mostly on the mandibles, with the incisivi (landmarks 18 to 23) being the most conspicuously asymmetric structures, as well as the insertion area of the mandible closer muscle (landmarks 28-29, 32-33; Fig. 3). DA can also be noticed in head structures which are located close to the mandibles, such as the clypeus-labrum region (landmarks 1-3 and 12), and the tentorial bridge (landmarks 14-15). The major directionally asymmetric patterns are the tilting of the clypeus-labrum region towards the right side, and the dorso-ventral and antero-posterior displacement of mandible incisivi. Fluctuating asymmetry (FA) is also significant, although its magnitude is much less than DA. FA is more spread out across the head than DA, however the incisivi also appear to be the structure with the larger magnitude for FA.

When using Neubauer et al.'s approach, it appears clearly that the major asymmetric component, explaining over 90% of asymmetric variance, is directional (Fig. 4). The second axis, which represents about 2% of asymmetric variance is centered around 0 (One sample t-test, mean = 0.0021, t = 0.5208, df = 48, P = 0.6049), and normally distributed (Shapiro-Wilk normality test, W = 0.9885, P = 0.9105), suggesting it represents an FA component. Further axes, representing ≤1% of asymmetric variance were not explored. Unexpectedly, iFA and iDA (i.e. positions of individuals along these two asymmetric axes) appear correlated with each other (Fig.4, Pearson's correlation, cor = 0.3569, t = 2.6191, df = 47, P = 0.0118). Similar results were obtained when restricting these analyses to landmarks from the mandibles only.

*Modularity and integration*. Both CR and EMMLi do not support the null hypothesis of no modularity in the grasshopper's head (Table 2, Supp. Mat. Table 1). EMMLi suggests the most likely modularity partition is the "Head-Mandibles asymmetric-Sensory" one (Fig. 2D), followed by the "Head-Mandibles asymmetric" one (Fig. 2C). On the other hand, the comparison between CR tests suggests the strongest modular signal is found in the simple 2 modules "Head-Mandibles" partition (Fig. 2A). CR suggests that all modular partitions are significantly different from the null hypothesis of no modularity, but also that differences between modular signals in all those partitions are not significantly different from each other (Table 2). It should also be noted that he partition with the second largest modular effect is the most complex model, the 4 modules "Head-Mandibles asymmetric-Sensory" partition. When running the comparison of CR tests again after module by module superimposition, the ZCR values get lower (i.e. modular signal gets stronger) overall. This is expected, as these "local" superimposition mathematically tend to add intra-module covariance, and reduce inter-module covariance. Again, the all partitions are significantly different from the null hypothesis of no modularity (all P < 0.001), and again the differences between partitions in terms of modularity signal are not significant (all P > 0.1). The strongest modular signal (lowest ZCR) is found, as with the global superimposition, in the 2 modules "Head-Mandibles" partition, and the 4 modules "Head-Mandibles asymmetric-Sensory" partition also has strong modular signal (Fig. 5). It is however striking that the "Half-Half" partition, which has the weakest modular signal with the global superimposition, has on the other hand the second strongest signal in the module by module superimposition analysis.

Table 1. Results from the bilateral object symmetry shape ANOVA (Type I), using 1000 Randomized Residual Permutations (RRPP) for significance testing.

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
|  | Df | SS | MS | Rsq | F | Z | Pr..F. |
| ind | 48 | 0.14689 | 0.00306 | 0.24838 | 2.6422 | -7.25098 | 1 |
| side | 1 | 0.3648 | 0.3648 | 0.61686 | 314.97456 | 3.36624 | 0.001 |
| ind:side | 48 | 0.05559 | 0.00116 | 0.09401 | 4.70964 | 14.47838 | 0.001 |
| ind:side:replicate | 98 | 0.0241 | 0.00025 | 0.04075 |  |  |  |
| Total | 195 | 0.59138 |  |  |  |  |  |

Table 2. Comparison of effect sizes (ZCR) from CR modularity analyses.

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | **A. Effect sizes (ZCR). Negative values represent stronger modular signal.** | | | | | | | |
|  | No Modules | Head-Mandibles | Head-Mandibles-Sensory | Head-Mandibles asymmetric | Head-Mandibles asymmetric-Sensory | Ventral-Dorsal | Half-Half | Mandibles only |
|  | 0 | -4.5 | -3.34 | -3.25 | -4.13 | -3.5 | -2.21 | -3.23 |
|  | **B. Pairwise differences in effect size.** | | | | | | | |
| No Modules | 0 | 4.5 | 3.34 | 3.25 | 4.13 | 3.5 | 2.21 | 3.23 |
| Head-Mandibles | 4.5 | 0 | 0.16 | 0.01 | 0.13 | 0.19 | 1.59 | 0.43 |
| Head-Mandibles-Sensory | 3.34 | 0.16 | 0 | 0.12 | 0.26 | 0.31 | 1.24 | 0.24 |
| Head-Mandibles asymmetric | 3.25 | 0.01 | 0.12 | 0 | 0.12 | 0.18 | 1.31 | 0.35 |
| Head-Mandibles asymmetric-Sensory | 4.13 | 0.13 | 0.26 | 0.12 | 0 | 0.07 | 1.61 | 0.52 |
| Ventral-Dorsal | 3.5 | 0.19 | 0.31 | 0.18 | 0.07 | 0 | 1.51 | 0.54 |
| Half-Half | 2.21 | 1.59 | 1.24 | 1.31 | 1.61 | 1.51 | 0 | 1.03 |
| Mandibles only | 3.23 | 0.43 | 0.24 | 0.35 | 0.52 | 0.54 | 1.03 | 0 |
|  | **C. Pairwise P-values associated with pairwise differences in effect size.** | | | | | | | |
| No Modules | 1 | 0 | 0 | 0 | 0 | 0 | 0.03 | 0 |
| Head-Mandibles | 0 | 1 | 0.88 | 0.99 | 0.9 | 0.85 | 0.11 | 0.67 |
| Head-Mandibles-Sensory | 0 | 0.88 | 1 | 0.9 | 0.79 | 0.76 | 0.22 | 0.81 |
| Head-Mandibles asymmetric | 0 | 0.99 | 0.9 | 1 | 0.91 | 0.86 | 0.19 | 0.72 |
| Head-Mandibles asymmetric-Sensory | 0 | 0.9 | 0.79 | 0.91 | 1 | 0.94 | 0.11 | 0.6 |
| Ventral-Dorsal | 0 | 0.85 | 0.76 | 0.86 | 0.94 | 1 | 0.13 | 0.59 |
| Half-Half | 0.03 | 0.11 | 0.22 | 0.19 | 0.11 | 0.13 | 1 | 0.31 |
| Mandibles only | 0 | 0.67 | 0.81 | 0.72 | 0.6 | 0.59 | 0.31 | 1 |