

# Ontogeny of individual asymmetries in several traits of larval *Chironomus riparius* Meigen, 1804 (Diptera, Chironomidae)

María J. Servia, Fernando Cobo, and Marcos A. González

**Abstract:** In recent years considerable effort has been dedicated to the analysis of alterations in the morphology of individuals, mainly fluctuating asymmetry, as bioindicators of stress. However, inconsistent results and a general lack of knowledge about the processes involved in the generation and development of asymmetries have discouraged some researchers from using this tool. We present a study of the ontogeny of individual asymmetries in several traits of *Chironomus riparius* larvae. Development of asymmetries through consecutive instars showed no evidence of sidedness and was in accordance with a compensatory growth model, since growth of the smaller side was typically greater than growth of the larger side during one moult. Surprisingly, however, although growth was compensatory, right–left (R–L) values were size-dependent and increased throughout larval development. Once R–L values were corrected for size dependence, asymmetry levels proved to be similar in all instars except for instar I in one case. We discuss our findings in relation to the developmental patterns and regulation mechanisms described and proposed in similar studies. Specifically, the coincidence of our results with those of another study on insects may indicate the existence of similar regulatory systems throughout this group of animals.

**Résumé :** Ces dernières années, beaucoup d'efforts ont été consacrés à l'analyse des modifications de la morphologie des individus comme bio-indicateurs du stress, particulièrement l'asymétrie fluctuante. Cependant, des résultats contradictoires et un manque de connaissance des processus impliqués dans la formation et le développement des asymétries ont amené des chercheurs à négliger cette méthodologie. On trouvera ici une étude de l'ontogénie d'asymétries individuelles de plusieurs caractères chez les larves de *Chironomus riparius*. Le développement des asymétries au cours des stades successifs de croissance n'indique aucune tendance à se produire d'un côté plutôt que de l'autre et se fait en accord avec un modèle de croissance compensatoire, puisque la croissance du côté le moins développé durant la mue est typiquement plus importante que celle du côté plus grand. Étonnamment, cependant, bien que la croissance soit compensatoire, les valeurs droite–gauche sont fonction de la taille et augmentent au cours du développement larvaire. Après une correction pour tenir compte de la taille, les niveaux d'asymétrie sont semblables chez tous les stades, excepté dans un cas au premier stade. Nos résultats sont examinés à la lumière des modèles de développement et des mécanismes de régulation décrits et proposés dans des études antérieures similaires. En particulier, la forte ressemblance entre nos résultats et ceux d'une autre étude sur les insectes peut indiquer l'existence de systèmes régulateurs semblables au sein de ce groupe d'animaux.

[Traduit par la Rédaction]

## Introduction

The final morphology of an individual depends on a delicate equilibrium of different physiological processes that occur during its growth and development. Alterations in the expected shape and size of any structure may be caused by several factors, such as different types of genetic and environmental stress that affect those developmental mechanisms (see the review in Møller and Swaddle 1997). As a consequence, alterations in the morphology of individuals (i.e., deformities, phenodeviations, asymmetries) are currently be-

ing used as bioindicator tools for detecting and assessing stress conditions (e.g., deformities: Servia et al. 1998; Burger and Snodgrass 2000; Meregalli et al. 2000; Pollet and Bendell-Young 2000; Prygiel et al. 2000; fluctuating asymmetry (FA): Rettig et al. 1997; Bleeker et al. 1999; Allenbach et al. 1999; deformities and FA: Groenendijk et al. 1998). Moreover, alterations in the morphology of an individual may have important or even dramatic consequences for its fitness (i.e., fecundity and (or) survival), therefore numerous studies have analysed relationships between morphological alterations and the phenotypic or genotypic quality of individuals (e.g., deformities: Janssens de Bisthoven et al. 1992, 1998; FA: Møller and Pomiankowski 1994; Watson and Thornhill 1994; Dufour and Weatherhead 1997; McLachlan 1997; Møller 1997, though see Clarke 1998).

Among the systems used as indicators of stress, one that has become increasingly popular is the analysis of FA (minor, nondirectional deviations from bilateral symmetry). The number of studies related to this subject is enormous (for references see Møller and Swaddle 1997), but recently some

Received 23 January 2002. Accepted 23 July 2002. Published on the NRC Research Press Web site at <http://cjz.nrc.ca> on 20 September 2002.

M.J. Servia,<sup>1</sup> F. Cobo, and M.A. González. Departamento de Biología Animal, Facultad de Biología, University of Santiago de Compostela, Campus Sur s/n, 15782 Santiago de Compostela, Spain.

<sup>1</sup>Corresponding author (e-mail: [baservia@usc.es](mailto:baservia@usc.es)).

authors have questioned its utility as a biomonitoring tool in view of our poor current knowledge of different aspects of FA–stress relationships, such as the influence of the type of character (e.g., sexual or nonsexual traits) on its response to stress (Rabitsch 1997; Bjorksten et al. 2000a), the magnitude of stress needed to detect an effect on FA (Dobrin and Corkum 1999), or the developmental basis of FA (Bjorksten et al. 2000b).

Although some theoretical background has been developed regarding the origins of FA (e.g., Emlen et al. 1993; Graham et al. 1993; Palmer 1996), a number of authors have suggested that studies in this area remain of key importance for FA analysis (Collin 1997; Hallgrímsson 1998; Van Dongen and Lens 2000). Indeed, studies on the ontogeny of individual asymmetries may provide new insights into the regulatory capabilities of individuals, since we still do not know how regulatory systems control the growth of both sides of a bilateral trait. Knowledge on this subject may also shed light on the inconsistent results of analyses of the FA–stress relationship. As pointed out by Tomkins (1999), “Even though such a diversity of traits (from feathers to forceps) could experience similar selective forces (e.g., preferences for size and symmetry), the developmental biology of each trait is likely to be very different, and this may contribute to the lack of concordance”.

Exuviae of insects are useful structures for monitoring the development of asymmetries over the development of a given individual. The chitinous nature of exuviae allows each cuticle cast to be recovered and accurately measured, providing a record of each growth period. Note, though, that the mechanisms regulating postembryonic growth in insects are still under discussion, mainly because of the limitations the premoult cuticle may impose on the growth of the new cuticle (Klingenberg 1996; Tomkins 1999).

In the present study, larvae of the midge *Chironomus riparius* (Diptera, Chironomidae) were selected because they are easily cultured under laboratory conditions. The four larval instars present a sclerotized head capsule that can be recovered after each moult. Thus, by means of a simple individual culture system we were able to obtain complete series of head capsules of the four larval instars, and could thus follow the development of individual asymmetries in several larval traits.

## Materials and methods

### Sample collection and culture of larvae

Egg masses of *C. riparius* were collected from a site on the River Sar near Bertamiráns in Galicia, northwestern Spain. This site is a short distance downstream from the wastewater-treatment plant that processes the urban and industrial wastes produced in the town of Santiago de Compostela. The plant is severely deficient, and this stretch of river is therefore characterized by a high organic-matter content. In addition, aeration is good, favouring the presence of chironomid larvae (notably *C. riparius* and *Prodiamesa olivacea*) throughout the year.

Nine egg masses were collected and transported to the laboratory in cooled jars. Once in the laboratory the egg masses were incubated at room temperature ( $22 \pm 2^\circ\text{C}$ ) in petri dishes filled with clean water (distilled water containing 35 mg/L NaCl, 2 mg/L  $\text{KH}_2\text{PO}_4$ , 61.5 mg/L  $\text{MgSO}_4$ ,

36 mg/L  $\text{CaCl}_2$ , 5 mg/L  $\text{NaHCO}_3$ , and 1.6 mg/L  $\text{FeCl}_3$ ). Petri dishes were checked twice a day for the hatching of the eggs. Once first-instar larvae hatched and left the egg mass, 300 larvae were individually transferred to small culture tubes (diameter 1.3 cm, height 1.5 cm) containing 1.5 mL of clean water. The culture tubes were numbered and placed in small white trays (10.5 × 10.5 cm) with holes for the tubes. Owing to the small dimensions of the trays and the tubes, it is possible to observe the larvae under a binocular microscope without disturbing them.

Just before the larvae were transferred to the tubes, a few small Tetramin<sup>®</sup> granules (diameter 100–500  $\mu\text{m}$ ) were added to each to provide the larvae with food and material for constructing cases. This initial addition was critical for the experiment because excessive food can deplete oxygen levels in the water and kill the larvae. Furthermore, if too many granules are added, it is more difficult to find the small exuvia of the first-instar larva when it moults to the second instar. Tetramin<sup>®</sup> is a commercial product for feeding fish but is frequently used as food in chironomid cultures because its composition is well suited for larval growth (Holloway 1983; Vos et al. 2000). It has been widely used in previous experimental studies of deformities of chironomid larvae (e.g., Hudson and Ciborowski 1996; Janssens de Bisthoven et al. 1997; Vermeulen 1998; Aston 1998), and it is generally accepted to have no teratogenic effects, although this has not been conclusively demonstrated.

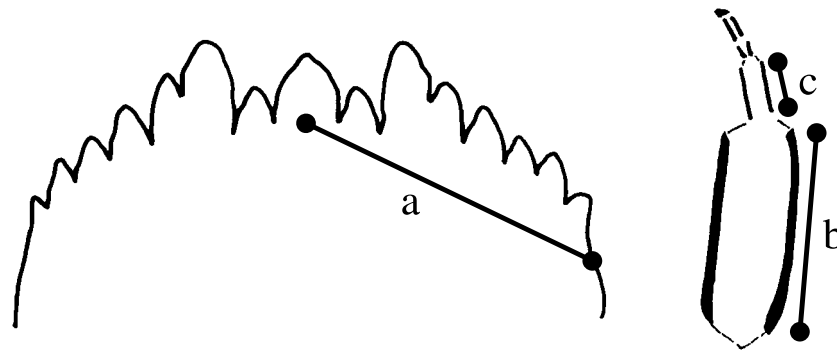
Tubes were checked every day and the head capsules of the exuviae from each instar were collected and mounted on microscope slides. After the larvae had moulted to the second instar, the water was replaced and new food added if necessary. The control was maintained until larvae reached the fourth instar, when individuals were killed and the head capsule was mounted after clearing in hot 8% KOH. All capsules were mounted ventral side up. When the experiment was finished, each slide contained the four head capsules of each individual. The total amount of Tetramin<sup>®</sup> each larva received over the experiment (i.e., between hatching and the fourth instar) was approximately 3–4 mg. This is thought to be adequate for normal larval growth in culture (Holloway 1983).

In total we obtained 237 complete series for all the structures (i.e., 237 slides on which the menta and antennae of the four capsules could be studied; see below). For measurements we selected 50 series in which no mounting distortions or any other artefact could be observed.

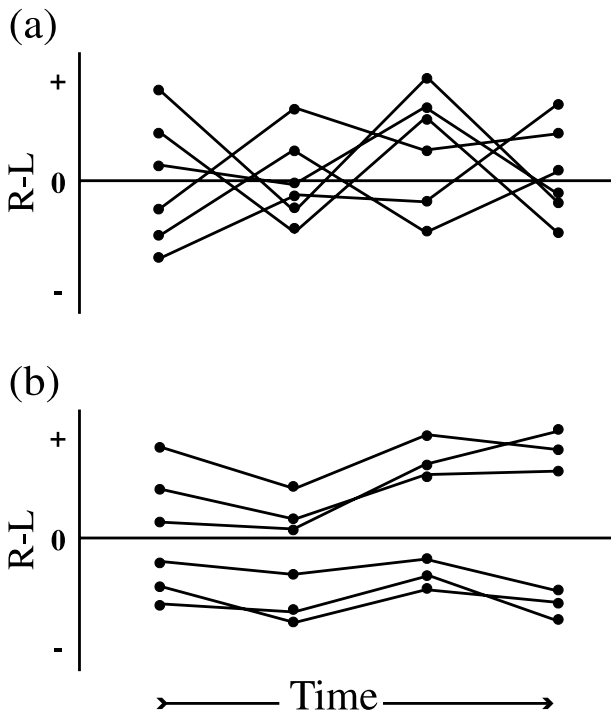
### Measurement protocol

The traits selected for monitoring individual asymmetries were the width of the mentum and the lengths of antennal segments I and II. These were the only traits that we were able to accurately measure in all larval instars (see Fig. 1) because first-instar larvae exhibit weak sclerotization. Microscope slides were observed at 400× or 200× magnification, depending on the instar, using a Olympus BX50 microscope equipped with a camera that took digital photographs (Olympus DP10, resolution 1 410 000 total pixels). Photographs were taken of all individuals so that the mentum and antennae took up most of the image. Mentum width and antennal-segment lengths were determined using an image-analysis system (Micro Image 4.0 for Windows). All measurements

**Fig. 1.** Landmarks used for measuring mentum width (a) and lengths of antennal segments I (b) and II (c).



**Fig. 2.** Changes in (R-L) values throughout the developmental period if sidedness exists (a) and does not exist (b). Each line represents an individual.

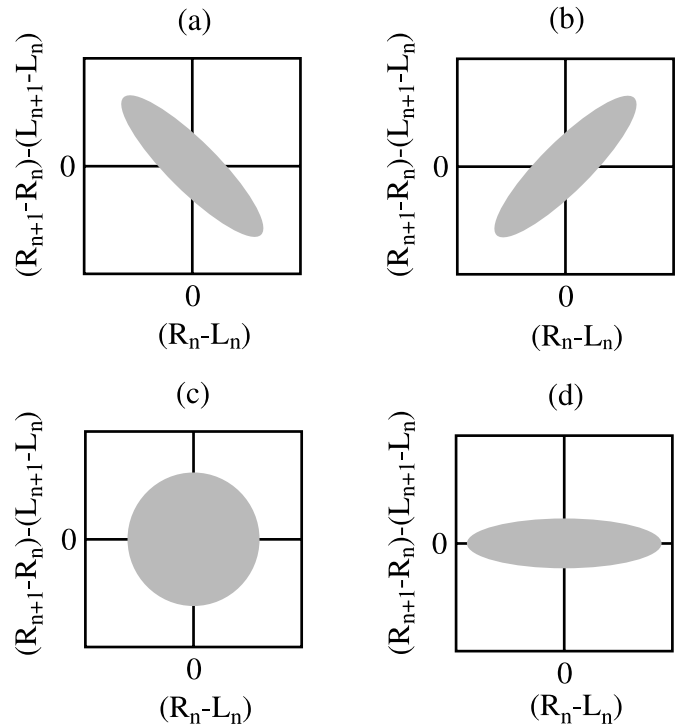


were taken twice from the same image, in random order each time. The data were automatically sent to a database, so the researcher had no knowledge of the first measurement when obtaining the second measurement. All measurements are given in micrometres.

**Measurement error**

Following Pither and Taylor (2000), two potential sources of measurement error were evaluated: human measurement and photographic procedure used. Human measurement error was assessed for each instar using a two-way ANOVA with *side* (fixed) and *individual* (random) as the two factors, as recommended by Palmer (1994). Before this test was performed, box plots of right-left asymmetry (R-L) data were screened for outliers, since such values may be related not to asymmetry but to physical damage or severe deformities (Palmer 1994). To evaluate error due to the procedure used for taking photographs, we selected 10 individuals from in-

**Fig. 3.** Expected correlations between (R-L) at instar  $n$  and growth of each side from instar  $n$  to instar  $n + 1$  ( $(R_{n+1}-R_n) - (L_{n+1}-L_n)$ ), assuming a compensatory growth model (a), a persistent growth model (b), and an uncorrelated growth model (c) (see Tomkins 1999), or assuming equal growth on the two sides (i.e., same size of asymmetries) (see Chippindale and Palmer 1994) (d).



stars I and IV and took one photograph of each. After two replicates of the measurements for each side (right and left) had been obtained, we took a second photograph of each individual and again obtained two replicate measurements (in random order in all cases). Data for each trait in each instar were analysed separately (2 instars  $\times$  3 traits = 6 tests) using a nested-factorial ANOVA with *photograph* and *individual* as random-effect factors (*photograph* nested within *individual*), *side* as factorial or crossed factor, and *repeated measurement* as the residual error (Hicks 1973). Having ruled out significant contributions of any source of error to our data, replicate measurements on the same side were averaged for further analysis.

**Table 1.** Results of the two-way ANOVA (*side* × *individual*) performed for each trait in each instar.

Trait	<i>n</i>	Mean square (ANOVA)			
		<i>Side</i>	Individual	<i>Side</i> × <i>individual</i>	<i>Error</i>
<b>Instar I</b>					
Mentum width	50	0.518	4.346***	1.038***	0.215
Antennal segment I length	50	0.692	1.401***	0.388**	0.203
Antennal segment II length	49	1.760	2.249***	0.464***	0.146
<b>Instar II</b>					
Mentum width	50	0.866	7.166***	7.467***	0.211
Antennal segment I length	50	4.767	9.827***	2.364***	0.136
Antennal segment II length	50	1.419	3.592***	0.978***	0.145
<b>Instar III</b>					
Mentum width	50	0.204	32.914***	5.375***	0.418
Antennal segment I length	50	6.883	62.222***	9.836***	1.073
Antennal segment II length	50	2.459	8.303***	3.143***	0.296
<b>Instar IV</b>					
Mentum width	50	17.943	115.384***	10.063***	1.289
Antennal segment I length	50	115.848	316.327***	33.754***	3.794
Antennal segment II length	49	6.270	24.224***	3.053***	1.004

**Note:** For *individual*, significance indicates that the differences among individuals are larger than the measurement error (*error*). For *side*, significance indicates the existence of directional asymmetry. For *side* × *individual*, significance indicates that nondirectional asymmetry is larger than the measurement error. \*\*, *P* < 0.01; \*\*\*, *P* < 0.001.

**Table 2.** Results of the nested-factorial ANOVA with factors *individual* (*n* = 10), *photograph* (*n* = 2; *photograph* nested within *individual*), and *side* (*n* = 2) performed for each trait in each instar.

Trait	Mean square (ANOVA)					<i>Error</i>
	<i>Side</i>	<i>Individual</i>	<i>Side</i> × <i>individual</i>	<i>Photograph</i> ( <i>individual</i> )	<i>Side</i> × <i>photograph</i> ( <i>individual</i> )	
<b>Instar I</b>						
Mentum width	3.683	7.006*	1.519**	0.381	156	0.152
Antennal segment I length	0.365	2.950*	0.740**	0.126	123	0.081
Antennal segment II length	1.173	2.203	0.924***	0.106	41	0.088
<b>Instar IV</b>						
Mentum width	0.081	188.120***	7.499***	0.628	264	0.525
Antennal segment I length	51.454	268.295**	37.476***	1.688	1412	0.867
Antennal segment II length	3.391	17.880	8.369***	0.522	643	0.349

**Note:** See the text for further details. \*, *P* < 0.05; \*\*, *P* < 0.01; \*\*\*, *P* < 0.001.

**Patterns of asymmetry in the measured traits**

Since the aim of the present study was to contribute to knowledge of regulatory systems when FA is present, other types of asymmetry must be ruled out. We thus examined our traits for the presence of directional asymmetry or antisymmetry in each instar, following the methods of Palmer (1994). Specifically, the significance of the factor *side* in the two-way ANOVA used to test for measurement error would reveal the existence of directional asymmetry, and deviations of the distribution of R-L from normality (skew and kurtosis) would serve to detect antisymmetry. Normality was tested using one-sample *t* tests and graphic techniques.

**Patterns of ontogenic evolution of asymmetries**

Possible mechanisms involved in the generation or amelioration of asymmetries have been put forward in the light of a number of empirical studies (Chippindale and Palmer

1994; Møller 1996; Collin 1997; Møller and Swaddle 1997; Swaddle and Witter 1997; Aparicio 1998; Tomkins 1999). We used methods developed in several of these studies.

An important question in this context is whether or not consistent sidedness (i.e., consistency in the direction of asymmetry from one moult to the next) exists. Such consistency implies that asymmetries are fixed early in larval development, and that the regulatory system is not capable of altering the direction of asymmetry (Møller 1996). This question can be studied by representing the signed values of asymmetry (R-L) of each trait in each instar or at each moment of measurement as a diagram (e.g., Collin 1997; Møller and Swaddle 1997; Swaddle and Witter 1997) (see Fig. 2). However, amelioration or magnification of asymmetries over time is difficult to assess using such diagrams, especially when the number of individuals is large. Tomkins (1999) studied the evolution of asymmetries in earwig, *Forficula auricularia*,

**Table 3.** Descriptive statistics for the selected traits.

Trait	n	(R + L)/2 (mean ± SE)	(R-L)		
			Mean ± SE	Skew ± SE	Kurtosis ± SE
<b>Instar I</b>					
Mentum width	50	20.380 ± 0.147	0.102 ± 0.144	0.205 ± 0.337	-0.462 ± 0.662
Antennal segment I length	50	10.610 ± 0.084	-0.118 ± 0.088	-0.087 ± 0.337	-0.085 ± 0.662
Antennal segment II length	49	14.583 ± 0.104	0.167 ± 0.107	-0.062 ± 0.340	-0.379 ± 0.668
<b>Instar II</b>					
Mentum width	50	32.465 ± 0.189	0.132 ± 0.178	-0.360 ± 0.337	-0.340 ± 0.662
Antennal segment I length	50	27.227 ± 0.222	0.309 ± 0.217	-0.051 ± 0.337	-0.307 ± 0.662
Antennal segment II length	50	13.444 ± 0.134	0.168 ± 0.140	-0.002 ± 0.337	-0.596 ± 0.662
<b>Instar III</b>					
Mentum width	50	55.704 ± 0.406	0.064 ± 0.328	-0.029 ± 0.337	1.005 ± 0.662
Antennal segment I length	50	52.334 ± 0.558	0.371 ± 0.444	0.488 ± 0.337	0.299 ± 0.662
Antennal segment II length	50	18.334 ± 0.204	-0.222 ± 0.251	0.011 ± 0.337	-0.105 ± 0.662
<b>Instar IV</b>					
Mentum width	50	94.870 ± 0.760	0.599 ± 0.449	0.177 ± 0.337	0.840 ± 0.662
Antennal segment I length	50	106.547 ± 1.258	1.522 ± 0.822	0.268 ± 0.337	-0.327 ± 0.662
Antennal segment II length	49	23.568 ± 0.351	0.572 ± 0.324	0.386 ± 0.340	0.095 ± 0.668

**Note:** (R + L)/2 is structure size (µm); (R-L) is right-left asymmetry. None of the values was statistically significant at the 5% level.

**Table 4.** Fluctuating-asymmetry indices for each larval instar.

Trait	n	R-L  (mean ± SE)	Mean  R-L /mean [(R + L)/2]	σ <sub>i</sub> <sup>2</sup>	df
<b>Instar I</b>					
Mentum width	50	0.794 ± 0.090	0.039	0.412	30.169
Antennal segment I length	50	0.487 ± 0.057	0.046	0.093	9.822
Antennal segment II length	49	0.601 ± 0.070	0.041	0.159	21.503
<b>Instar II</b>					
Mentum width	50	1.020 ± 0.103	0.031	0.684	36.453
Antennal segment I length	50	1.264 ± 0.129	0.046	1.114	43.454
Antennal segment II length	50	0.812 ± 0.082	0.060	0.417	35.169
<b>Instar III</b>					
Mentum width	50	1.649 ± 0.228	0.030	2.479	41.552
Antennal segment I length	50	2.424 ± 0.282	0.046	4.382	38.667
Antennal segment II length	50	1.410 ± 0.153	0.077	1.424	40.031
<b>Instar IV</b>					
Mentum width	50	2.467 ± 0.290	0.026	4.387	36.954
Antennal segment I length	50	4.846 ± 0.493	0.045	14.980	38.366
Antennal segment II length	49	1.556 ± 0.241	0.066	1.025	20.533

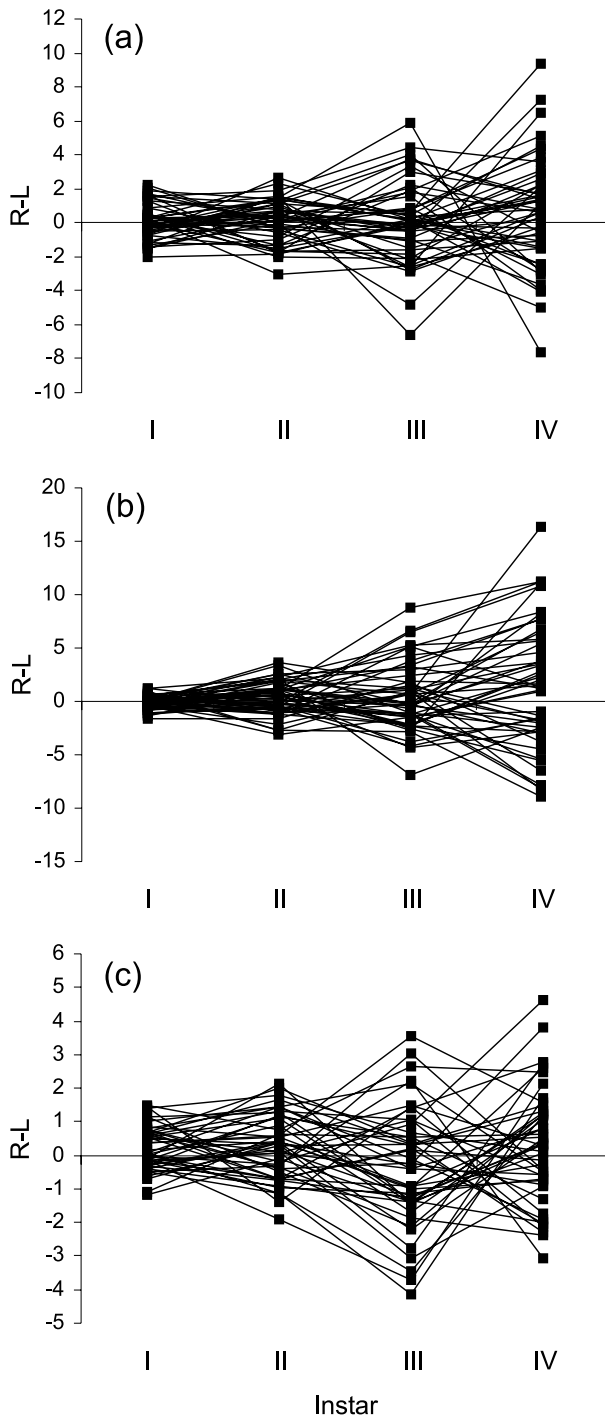
**Note:** |R-L| shows absolute values of R-L (FA1; Palmer 1994). Mean |R-L|/mean [(R + L)/2] shows asymmetry of the sample corrected for mean trait size (FA3; Palmer 1994); σ<sub>i</sub><sup>2</sup> is an index calculated by two-way ANOVA (*side* × *individual*) indicating between-sides variance once measurement error has been eliminated (FA10; Palmer 1994); and df shows approximate degrees of freedom for FA10.

forceps over time by examining the relationships between asymmetries in one instar (R<sub>n</sub>-L<sub>n</sub>) and growth of both sides from that instar to the next (R<sub>n+1</sub>-R<sub>n</sub>) - (L<sub>n+1</sub>-L<sub>n</sub>). He proposed three growth models, compensatory growth (Fig. 3a), persistent growth (Fig. 3b), and uncorrelated growth (Fig. 3c), based on the existence of negative or positive covariance between asymmetry and growth (compensatory and persistent) or the lack of such a relationship (uncorrelated). Chippindale and Palmer (1994) obtained evidence of another model applicable to the development of asymmetries in crabs (see the Discussion though): constant growth on both sides (Fig. 3d).

Another important factor in studying asymmetries is the time course of their magnitudes over the whole developmen-

tal process in relation to the time course of the size of the corresponding structure. Thus, real asymmetries in a given structure may increase from one instar to the next, but the magnitude of the increment may or may not be related to the magnitude of the corresponding increment in the size of the structure. We tested whether asymmetry was dependent on structure size at each instar by using regression analysis and visual inspection of plots of log[*var*(R-L)] versus mean [(R + L)/2] (Palmer 1994). In cases where such a relation was observed, relative asymmetries (real asymmetries corrected for structure size: |R-L|/[(R + L)/2]) were calculated and differences among instars tested by means of a repeated-measures one-way ANOVA for each trait (factor instar) after

**Fig. 4.** Comparative diagrams of real changes in (R-L) for mentum width (a), antennal segment I length (b), and antennal segment II length (c) from the first to the fourth instar.



Box-Cox transformation of the data (Swaddle et al. 1994). All tests were run using SPSS (version 10.0).

**Results**

**Measurement error**

Outliers were detected in two cases for antennal segment II: one in a first-instar larva and another in a fourth-instar larva. Antennal segment II lengths for both individuals were

thus excluded from the analysis. Two-way ANOVA (*side* × *individual*) performed for each trait in each instar revealed that human measurement error was always significantly smaller than nondirectional asymmetry (Table 1). Moreover, tests for the combined effect of photographic-procedure and human measurement error revealed that *photograph* had no significant effect in any of the tests (see Table 2). Since measurement error and the influence of photographic procedure were negligible for both first- and fourth-instar larvae, we consider that the measurements obtained from second- and third-instar larvae are also reliable.

**Patterns of asymmetry in the measured traits**

All tests indicated that the asymmetry in our traits was FA rather than directional asymmetry or antisymmetry (Tables 1 and 3).

**Patterns of ontogenic evolution of asymmetries**

FA indices for larval instars I-IV are listed in Table 4. Visual inspection of the plots of signed values of asymmetry (R-L) for each trait indicates that none of the traits shows consistent sidedness, since the signs of asymmetries change frequently from one instar to the next (Fig. 4).

Regressions of the values of  $(R_{n+1}-R_n) - (L_{n+1}-L_n)$  on the asymmetries  $(R_n-L_n)$  showed that in all cases the relationship was negative (Fig. 5), indicating the occurrence of compensatory growth. All regressions were statistically significant except for antennal segment I between instars III and IV.

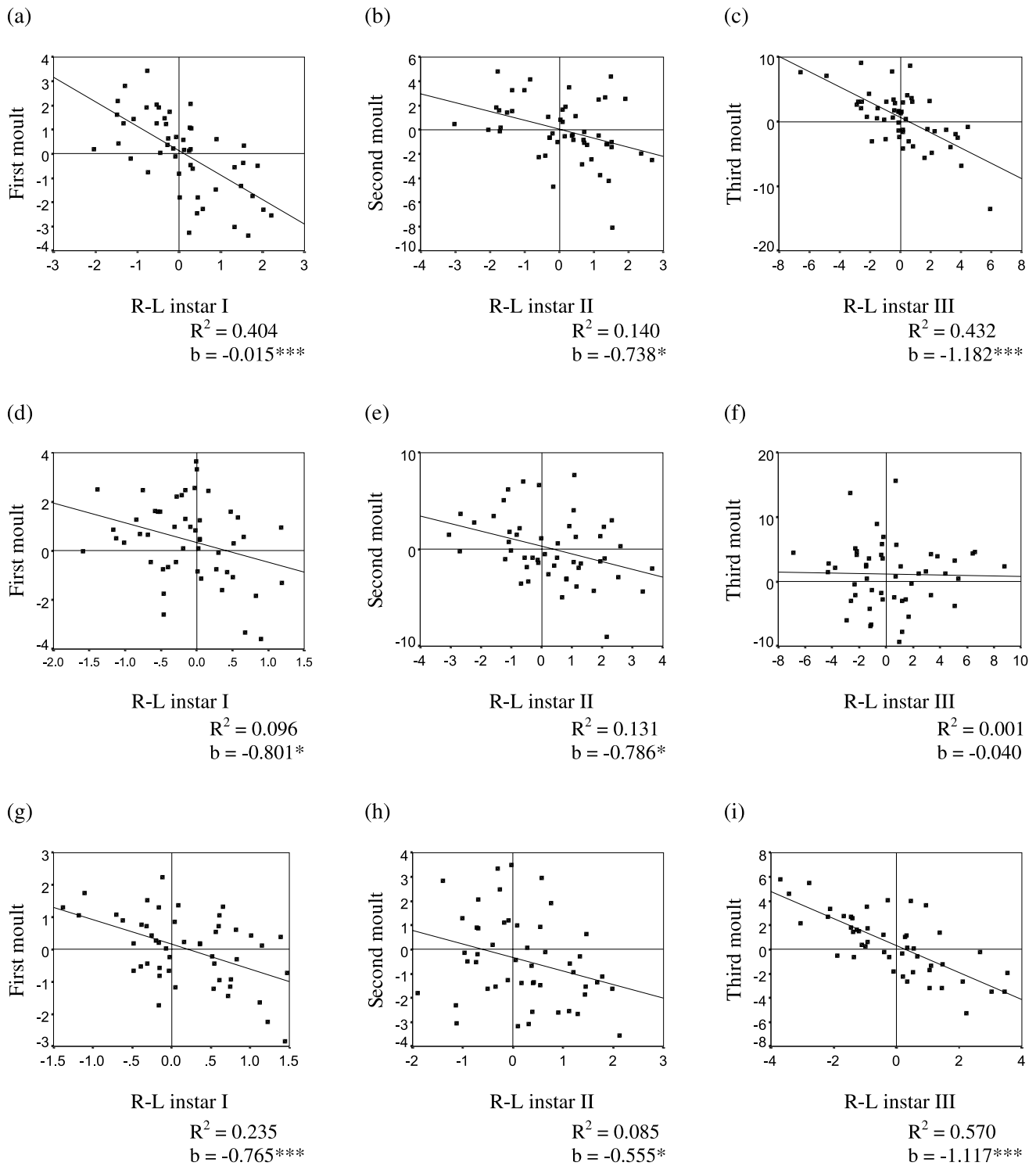
Regarding the dependence of asymmetries on structure size, regression of  $\log[\text{var}(R-L)]$  on mean  $[(R + L)/2]$  showed that this relationship exists for all the traits considered, though it was statistically significant at the 5% level only for mentum width (Fig. 6). Once asymmetry values were corrected for trait size and normalized using Box-Cox transformation, repeated-measures ANOVAs were performed for each trait (Table 5). Results show that relative asymmetries in mentum width and antennal segment I length do not differ among instars, but that they do differ for antennal segment II length (Table 5). Pairwise comparisons for the factor *instar* using Bonferroni correction (Rice 1989) show that differences exist between instars I and III. However, it should be noted also that pairwise comparisons using no correction show differences for mentum width between instars I and IV and for antennal segment II length between instar I and instars II, III, and IV, which suggests that instar I might present special characteristics.

**Discussion**

Studies of the development of morphological asymmetries have given rise to several theories of their origin and ontogeny (see reviews in Møller and Swaddle 1997; Swaddle and Witter 1997; Aparicio 1998; Hallgrímson 1998). These theories have been based upon two types of analysis: (1) analysis of structures showing continuous growth, with measurements of asymmetry obtained at regular intervals (generally every few days), and (2) analysis of structures that undergo regular moults, and in which an asymmetry value is obtained for each moult.

In studies of the first type, diverse results have been

**Fig. 5.** Relationship between (R-L) of mentum width ( $a-c$ ), antennal segment I length ( $d-f$ ) and antennal segment II length ( $g-i$ ) at instar  $n$  and growth of each side from instar  $n$  to instar  $n + 1$  ( $(R_{n+1}-R_n) - (L_{n+1}-L_n)$ ). The negative slope confirms compensatory growth. Positive values on the y axis indicate greater right-side than left-side growth during the moult; negative values indicate greater left-side than right-side growth (\*,  $P < 0.05$ ; \*\*\*,  $P < 0.001$ ).



obtained and diverse hypotheses put forward to explain them. Swaddle and Witter (1997) found that the direction of right-left asymmetry in primary feather length in starlings (*Sturnus vulgaris*) changes continuously during development, while

the absolute magnitude of asymmetry declines as the feathers reach their maximum length. These authors therefore suggested that compensatory growth mechanisms were acting. However, Aparicio (1998), who observed similar patterns in

**Table 5.** Results of the repeated-measures ANOVA (the source of variation is *instar*) for each trait.

Trait	df	Mean square	F
Mentum width			
Sphericity assumed	3	0.020	2.126
Error	138	0.009	
Antennal segment I length			
Greenhouse–Geisser adjustment	2.520	0.003	0.288
Error	113.421	0.011	
Huynh–Feldt adjustment	2.682	0.003	0.288
Error	120.702	0.010	
Antennal segment II length			
Sphericity assumed	3	0.063	5.547**
Error	138	0.011	

**Note:** \*\*,  $P < 0.01$ . For antennal segment II length, differences were found between instars I and III after pairwise comparison using Bonferroni correction. See the text for further details.

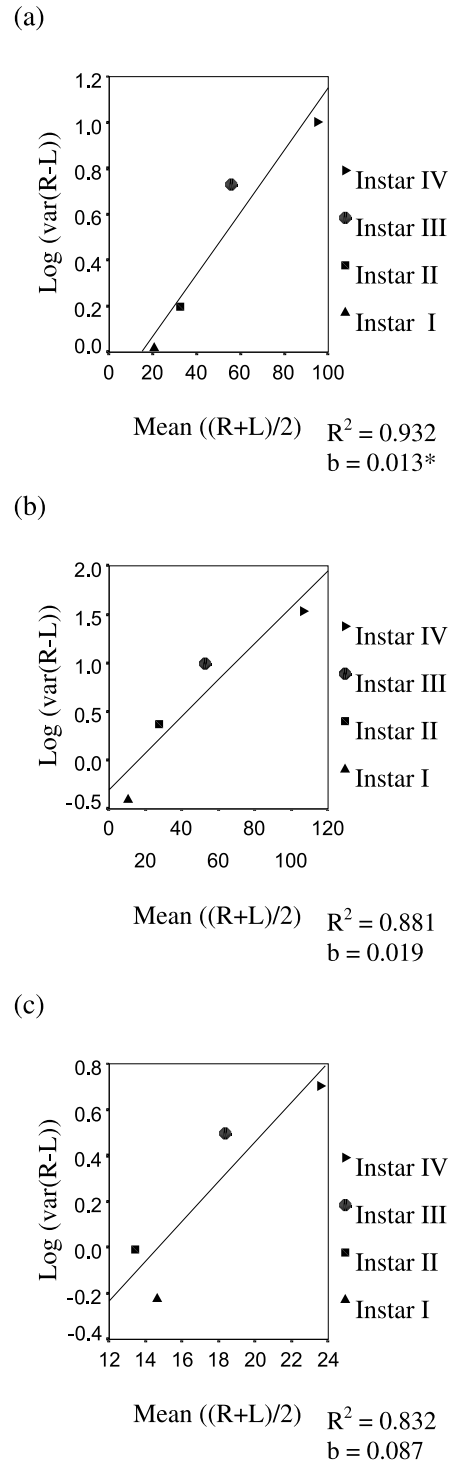
house sparrows (*Passer domesticus*), offers a different explanation: variability in feather length declines as the feathers approach their maximum size.

Collin (1997) obtained different results in a study of the development of different components of the larval skeleton of the irregular echinoderm *Dendraster excentricus*: specifically, the degree of asymmetry increased as the components grew. Furthermore, this author detected no clear trend in the development of asymmetry of the various components, and thus suggested that compensatory growth mechanisms are probably not acting in this species.

Despite the fact that the tendencies in the development of asymmetry values observed by Swaddle and Witter (1997) and Collin (1997) are contradictory, both studies included plots which clearly showed that the direction of asymmetry fluctuates over time. Møller and Swaddle (1997) offer the same type of representation of the values obtained in a study of *Ulmus glabra* leaves, in this case giving a totally different pattern, since the observed deviations increased and maintained their direction throughout the growth process. Those authors point out that this result may be explained by the nonlinear dynamic-feedback growth models proposed by a number of authors (Emlen et al. 1993; Graham et al. 1993), which have the effect of magnifying errors over time.

Similarly diverse results have been obtained in studies of organisms that undergo periodic moults. Chippindale and Palmer (1994) studied the development of right–left asymmetry in the segments of the appendices of the crab *Hemigrapsus nudus*. To this end, those authors raised crabs in isolation and collected exuviae at each moult. They found that asymmetry values were correlated between consecutive moults, in accordance with the model summarized in Fig. 3d, in which  $(R_{n+1}-R_n) - (L_{n+1}-L_n)$  values are always close to zero, since the two sides show the same growth. However, Tomkins (1999) has suggested that the method used by those authors is not appropriate for this type of study because, although the growth model does not depend in any way on asymmetry values (uncorrelated growth model), the existence of positive correlations between instar values and the next value may be due to ontogenic autocorrelation (see Klingenberg 1996). As

**Fig. 6.** Relationship between asymmetry and trait size at each instar for mentum width (a) and lengths of antennal segments I (b) and II (c). \*,  $P < 0.05$ .



noted, Tomkins (1999) suggests that the most reliable method for studying the development of asymmetries is to monitor growth on each side. This author studied asymmetry in the forceps of the dermapteran *F. auricularia* and found that in two of the three moults, growth closely fitted a compensatory growth model, with growth at the final moult consistent with

uncorrelated growth. Furthermore, the author indicates that the compensation was not perfect, since despite the fact that asymmetry values (relative to structure size) declined from early to late moults, absolute asymmetry values increased.

In the present study, the results obtained fully coincide with those of Tomkins (1999), except that the relative asymmetries remained more or less constant throughout development. This constancy may be related to the existence of chaotic attractors, which confer a certain stability during morphogenesis, although a nonlinear dynamical system confers stability even when it is not behaving chaotically (see Graham et al. 1993). The only exception to this constancy would be cases in which FA levels in instar I differ from those in instar IV for the mentum width and those in the rest of the instars for antennal segment II length, variations that may be due to the different morphological characteristics of instar I with respect to subsequent instars, implying that major morphological changes occur at this first moult. It would therefore be interesting to investigate whether rearing individuals under highly stressful conditions (or at least more stressful conditions than those of the present study) produces similar results or, by contrast, marked alterations in constancy.

The constancy in relative asymmetry levels observed in the present study contrasts with the results obtained in a study of mammal skeletons by Hallgrímson (1998), who found that relative asymmetry increases with the time required for development, and suggested that this may be due to accumulation of errors during development of the bone in question. However, as suggested above, it is very possible that different characters are governed by different regulatory systems, therefore it is fully possible that the systems governing the development of bone asymmetry in mammals are entirely different from those governing the development of asymmetry in arthropod structures.

That organisms like insects can compensate for asymmetries was regarded by Tomkins (1999) as a surprising phenomenon, since the new cuticle must use the old cuticle as a mould (Bennet-Clark 1971). However, Smith and Palmer (1994) have demonstrated that the crab *Cancer productus* can increase or decrease the size difference between the two pincers at each moult, depending on use. Furthermore, as we have noted in studies of deformities in *C. riparius*, at each moult not only minor abnormalities but also major deformities may be repaired (Servia 2001); if such dramatic repairs can occur, it seems reasonable to expect minor compensations in asymmetry also. Likewise, it is widely accepted nowadays that the insect cuticle is not an inert protective covering, but rather a living structure in close and continuous contact not only with epidermal cells but also with other organs (Csikós et al. 1999).

Some authors have demonstrated the existence of different systems regulating the growth of symmetric structures or regions. Such systems may be nervous, like those governing development of the claws of certain species of crab (Mellon and Stephens 1978; Govind and Pearce 1986), or biochemical via some sort of growth factor, like that governing the development of the wings of the butterfly *Precis coenia* (Klingenberg and Nijhout 1998). Thus, asymmetry within a given organism, or even of different structures within a sin-

gle organism, may be governed by different systems. This would at least partially explain the diversity of results obtained in studies of the ontogeny of asymmetries. In this respect it is particularly interesting that the results obtained for all structures considered in the present study coincide with those obtained by Tomkins (1999) in *F. auricularia*, raising the possibility that similar regulatory systems exist throughout the class Insecta.

## Acknowledgements

We thank Dr. J.C. Otero (University of Santiago de Compostela) for the use of his image-analysis system. This work was supported by project PGIDT01PX120002PR from the Xunta de Galicia. During the study M.J. Servia was in receipt of a scholarship from the Diputación Provincial de A Coruña. We are very grateful also to two anonymous reviewers whose constructive comments notably improved the manuscript.

## References

- Allenbach, D.M., Brown-Sullivan, K., and Lydy, M.J. 1999. Higher fluctuating asymmetry as a measure of susceptibility to pesticides in fishes. *Environ. Toxicol. Chem.* **18**: 899–905.
- Aparicio, J.M. 1998. Patterns of fluctuating asymmetry in developing primary feathers: a test of the compensational growth hypothesis. *Proc. R. Soc. Lond. B Biol. Sci.* **265**: 2353–2357.
- Aston, W.A. 1998. Morphological deformities in chironomid larvae (Chironomidae: Diptera): biomarkers of urban polluted sediments. Ph.D. thesis, Staffordshire University, England.
- Bennet-Clark, H.C. 1971. The cuticle as a template for growth in *Rhodnius prolixus*. *J. Insect Physiol.* **17**: 2421–2434.
- Bjorksten, T., David, P., Pomiankowski, A., and Fowler, K. 2000a. Fluctuating asymmetry of sexual and nonsexual traits in stalk-eyed flies: a poor indicator of developmental stress and genetic quality. *J. Evol. Biol.* **13**: 89–97.
- Bjorksten, T.A., Fowler, K., and Pomiankowski, A. 2000b. Symmetry, size and stress (reply). *Trends Ecol. Evol.* **15**: 331.
- Bleeker, E.A.J., Leslie, H.A., Groenendijk, D., Plans, M., and Admiraal, W. 1999. Effects of exposure to azaarenes on emergence and mouthpart development in the midge *Chironomus riparius* (Diptera: Chironomidae). *Environ. Toxicol. Chem.* **18**: 1829–1834.
- Burger, J., and Snodgrass, J.W. 2000. Oral deformities in several species of frogs from the Savannah River Site, USA. *Environ. Toxicol. Chem.* **19**: 2519–2524.
- Chippindale, A.K., and Palmer, A.R. 1994. Persistence of subtle departures from symmetry over multiple molts in individual brachyuran crabs: relevance to developmental stability. *In Developmental instability: its origins and evolutionary implications. Edited by T.A. Markow. Kluwer, Dordrecht, the Netherlands.* pp. 187–201.
- Clarke, G.M. 1998. Developmental stability and fitness: the evidence is not quite so clear. *Am. Nat.* **152**: 762–766.
- Collin, R. 1997. Ontogeny of subtle skeletal asymmetries in individual larvae of the sand dollar *Dendraster excentricus*. *Evolution*, **51**: 999–1005.
- Csikós, G., Molnár, D., Borhegyi, N.H., Talián, G.C., and Sass, M. 1999. Insect cuticle, an *in vivo* model of protein trafficking. *J. Cell Sci.* **112**: 2113–2124.
- Dobrin, M., and Corkum, L.D. 1999. Can fluctuating asymmetry in adult burrowing mayflies (*Hexagenia rigida*, Ephemeroptera) be

- used as a measure of contaminant stress? *J. Gt. Lakes Res.* **25**: 339–346.
- Emlen, J.M., Freeman, D.C., and Graham, J.H. 1993. Nonlinear growth dynamics and the origin of fluctuating asymmetry. *Genetica*, **89**: 77–96.
- Govind, C.K., and Pearce, J. 1986. Differential reflex activity determines claw and closer muscle asymmetry in developing lobsters. *Science (Washington, D.C.)*, **233**: 354–356.
- Graham, J.H., Freeman, D.C., and Emlen, J.M. 1993. Antisymmetry, directional asymmetry, and dynamic morphogenesis. *Genetica*, **89**: 121–137.
- Groenendijk, D., Zeinstra, L.W.M., and Postma, J.F. 1998. Fluctuating asymmetry and mentum gaps in populations of the midge *Chironomus riparius* (Diptera: Chironomidae) from a metal-contaminated river. *Environ. Toxicol. Chem.* **17**: 1999–2005.
- Hallgrímsson, B. 1998. Fluctuating asymmetry in the mammalian skeleton: evolutionary and developmental implications. *Evol. Biol.* **30**: 187–251.
- Hicks, C.R. 1973. *Fundamental concepts in the design of experiments*. Holt, Rinehart and Winston, New York.
- Holloway, M.T.P. 1983. Factors controlling the productivity of a benthic detritivore (*Chironomus riparius*). Ph.D. thesis, University of Wales, U.K.
- Hudson, L.A., and Ciborowski, J.J.H. 1996. Teratogenic and genotoxic responses of larval *Chironomus salinarius* group (Diptera: Chironomidae) to contaminated sediment. *Environ. Toxicol. Chem.* **15**: 1375–1381.
- Janssens de Bisthoven, L., Timmermans, K.R., and Ollevier, F. 1992. The concentration of cadmium, lead, copper and zinc in *Chironomus* gr. *thummi* larvae (Diptera, Chironomidae) with deformed versus normal menta. *Hydrobiologia*, **239**: 141–149.
- Janssens de Bisthoven, L., Huysmans, C., Vannevel, R., Goemans, G., and Ollevier, F. 1997. Field and experimental morphology response of *Chironomus* larvae (Diptera, Nematocera) to xylene and toluene. *Neth. J. Zool.* **47**: 227–239.
- Janssens de Bisthoven, L., Nuyts, P., Goddeeris, B., and Ollevier, F. 1998. Sublethal parameters in morphologically deformed *Chironomus* larvae: clues to understanding their bioindicator value. *Freshw. Biol.* **39**: 179–191.
- Klingenberg, C.P. 1996. Individual variation of ontogenies: a longitudinal study of growth and timing. *Evolution*, **50**: 2412–2428.
- Klingenberg, C.P., and Nijhout, H.F. 1998. Competition among growing organs and developmental control of morphological asymmetry. *Proc. R. Soc. Lond. B Biol. Sci.* **265**: 1135–1139.
- McLachlan, A. 1997. Size or symmetry: an experiment to determine which of the two accounts for mating success in male midges. *Ecoscience*, **4**: 454–459.
- Mellon, D., and Stephens, P.J. 1978. Limb morphology and function are transformed by contralateral nerve section in snapping shrimps. *Nature (Lond.)*, **272**: 246–248.
- Meregalli, G., Vermeulen, A.C., and Ollevier, F. 2000. The use of chironomid deformation in an *in situ* test for sediment toxicity. *Ecotoxicol. Environ. Saf.* **47**: 231–238.
- Møller, A.P. 1996. Development of fluctuating asymmetry in tail feathers of the barn swallow *Hirundo rustica*. *J. Evol. Biol.* **9**: 677–694.
- Møller, A.P. 1997. Developmental stability and fitness: a review. *Am. Nat.* **149**: 916–932.
- Møller, A.P., and Pomiankowski, A. 1994. Fluctuating asymmetry and sexual selection. *In* *Developmental instability: its origins and evolutionary implications*. Edited by T.A. Markow. Kluwer, Dordrecht, the Netherlands. pp. 269–281.
- Møller, A.P., and Swaddle, J.P. 1997. *Asymmetry, developmental stability and evolution*. Oxford University Press, Oxford.
- Palmer, A.R. 1994. Fluctuating asymmetry analyses: a primer. *In* *Developmental instability: its origins and evolutionary implications*. Edited by T.A. Markow. Kluwer Publishers, Dordrecht, the Netherlands. pp. 335–364.
- Palmer, A.R. 1996. Waltzing with asymmetry. *BioScience*, **46**: 518–532.
- Pither, J., and Taylor, P.D. 2000. Directional and fluctuating asymmetry in the black-winged damselfly *Calopteryx maculata* (Beauvois) (Odonata: Calopterygidae). *Can. J. Zool.* **78**: 1740–1748.
- Pollet, I., and Bendell-Young, L.I. 2000. Amphibians as indicators of wetland quality in wetlands formed from oil sands effluent. *Environ. Toxicol. Chem.* **19**: 2589–2597.
- Prygiel, J., Rosso-Darmet, A., Lafont, M., Lesniak, C., Durbec, A., and Ouddane, B. 2000. Use of oligochaete communities for assessment of ecotoxicological risk in fine sediment of rivers and canals of the Artois–Picardie water basin (France). *Hydrobiologia*, **410**: 25–37.
- Rabitsch, W.B. 1997. Levels of asymmetry in *Formica pratensis* Retz. (Hymenoptera, Insecta) from a chronic metal-contaminated site. *Environ. Toxicol. Chem.* **16**: 1433–1440.
- Rettig, J.E., Fuller, R.C., Corbett, A.L., and Getty, T. 1997. Fluctuating asymmetry indicates levels of competition in an even-aged poplar clone. *Oikos*, **80**: 123–127.
- Rice, W.R. 1989. Analyzing tables of statistical tests. *Evolution*, **43**: 223–225.
- Servia, M.J. 2001. Causalidad, ontogenia y aplicación práctica en la detección del estrés ambiental de la asimetría fluctuante y las deformidades en larvas de *Chironomus riparius* Mg. (Diptera: Chironomidae) de ecosistemas acuáticos de Galicia. Ph.D. thesis, University of Santiago de Compostela, Santiago de Compostela, Spain.
- Servia, M.J., Cobo, F., and González, M. 1998. Deformities in larval *Prodiamesa olivacea* (Meigen, 1818) (Diptera, Chironomidae) and their use as environmental stress indicators. *Hydrobiologia*, **385**: 153–162.
- Smith, L.D., and Palmer, A.R. 1994. Effects of manipulated diet on size and performance of brachyuran crab claws. *Science (Washington, D.C.)*, **264**: 710–712.
- Swaddle, J.P., and Witter, M.S. 1997. On the ontogeny of developmental stability in a stabilized trait. *Proc. R. Soc. Lond. B Biol. Sci.* **264**: 329–334.
- Swaddle, J.P., Witter, M.S., and Cuthill, I.C. 1994. The analysis of fluctuating asymmetry. *Anim. Behav.* **48**: 986–989.
- Tomkins, J.L. 1999. The ontogeny of asymmetry in earwig forceps. *Evolution*, **53**: 157–163.
- Van Dongen, S., and Lens, L. 2000. Symmetry, size and stress. *Trends Ecol. Evol.* **15**: 330–331.
- Vermeulen, A.C. 1998. Head capsule deformation in *Chironomus riparius* larvae (Diptera): causality, ontogenesis and its application in biomonitoring. Ph.D. thesis, Royal Belgian Institute of Natural Sciences, Brussels.
- Vos, J.H., Ooijevaar, M.A.G., Postma, J.F., and Admiraal, W. 2000. Interaction between food availability and food quality during growth of early instar chironomid larvae. *J. N. Am. Benthol. Soc.* **19**: 158–168.
- Watson, P.J., and Thornhill, R. 1994. Fluctuating asymmetry and sexual selection. *Trends Ecol. Evol.* **9**: 21–25.