

Persistence of subtle departures from symmetry over multiple molts in individual brachyuran crabs: relevance to developmental stability

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Abstract

Fluctuating asymmetry (FA) is generally viewed as a population-level character. It is described by some measure of the variance of the difference between the right and left sides for a collection of individuals. Very little is known of the developmental origins of FA, despite the fact that FA is widely used to estimate *developmental* stability. We present a novel technique for examining the growth trajectory of the asymmetries that give rise to FA, and we explore two sample data sets for the brachyuran crab *Hemigrapsus nudus*. We have traced the fate of these small, random deviations from perfect symmetry through three successive molts of individual crabs. Invertebrates that molt, and hence grow in discrete steps, provide an easily preserved record of their growth. Model II regressions of measurements from one molt versus the previous molt can help describe the stability of subtle departures from symmetry over time. Although any number of different patterns may occur, we identify four general cases: a) asymmetries vary at random throughout growth (random determination), b) asymmetries remain unchanged in sign and magnitude (constant asymmetries), c) asymmetries increase in proportion to character size and hence increase with growth (size-dependent asymmetries), and d) asymmetries persist, but are reduced in magnitude (damped asymmetries). Data from ten *Hemigrapsus nudus*, measured for between 21 and 28 metrical, limb-segment characters over three successive molts, yielded associations most similar to our pattern 'b', although some subtle departures in the direction of pattern 'c' were also observed. Persistent asymmetries accounted for 26% and 20% of the variance among asymmetries between molts 1 and 2, and molts 2 and 3 respectively. Thus, in spite of large and rapid increments in the external size of the crab, these subtle asymmetries tended to persist in both direction and magnitude, from molt to molt. This result suggests either i) that individual crabs have a genetic predisposition towards asymmetry in a particular direction but contribute to a continuous and normal distribution of *R-L* differences at the population level, or ii) that these subtle asymmetries arose at some earlier ontogenetic stage and were preserved through growth. Either interpretation has important ramifications for the study of FA. The first suggests that under some circumstances FA may not provide a valid measure of developmental instability, because subtle departures from symmetry in an individual may have a genetic basis. The second implies that subtle departures from bilateral symmetry are not 'corrected' as an individual grows.

Introduction

Fluctuating asymmetry (FA) has been used extensively as measure of developmental stability (Palmer & Strobeck, 1986; Leary & Allendorf,

1989; Parsons, 1990; Zakharov, 1992). The basis of this inference seems straightforward: if the expression of morphological characters on opposite sides of an individual organism is controlled by the same genes, then small, random deviations from perfect

bilateral symmetry that give rise to FA should provide a measure of the fidelity of development (developmental stability). Studies of FA over the past 40 years generally fall into two categories: 1) those using FA as a measure of extrinsic environmental stress such as temperature, food deprivation, pesticides, pollution, etc. (Zakharov, 1992) and 2) those using FA as a measure of intrinsic, or 'epigenetic', stress resulting from intense selection, inbreeding depression or hybrid disjunction (Palmer & Strobeck, 1986).

FA refers to one of several patterns of bilateral variation (VanValen, 1962; Palmer & Strobeck, 1992). FA is generally described statistically as a variance of signed, or a mean of unsigned, differences between sides of a sample of individuals (Palmer & Strobeck, 1986). Thus FA is defined as a statistical attribute of a sample of individuals and is not an attribute of an individual organism (VanValen, 1962). To try to avoid confusion, we will use the phrase 'subtle asymmetries' to refer to those departures from symmetry in a given individual that, when examined for a sample of individuals, would yield a pattern of ideal FA. Subtle asymmetries may potentially arise as a result of genetic, environmental, or genotype by environment effects on development (Palmer, Strobeck & Chippindale, 1993), although most appear to arise from developmental noise (Zakharov, 1992).

Although some studies have explored genetic aspects of FA (Mather, 1953; Leamy, 1984; Coyne, 1987; Livshits *et al.*, 1988; McKenzie & Clarke, 1988; Tuinstra, Dejong & Scharloo, 1990), studies exploring the ontogeny or persistence throughout growth of subtle asymmetries are conspicuously absent from a literature on *developmental stability* (see, however, Price *et al.*, 1991). When are subtle asymmetries determined during ontogeny? How is their magnitude related to body size or stage of development? Do they actually fluctuate as an individual grows? Are subtle departures from bilateral symmetry corrected during growth?

We present a novel approach for examining subtle asymmetries ontogenetically or throughout growth. Quite simply, if measurements are non-destructive, the asymmetry of a single character on a single individual may be tracked through time. We applied this method to a growth series of a common shore crab of the northeastern Pacific, *Hemigrapsus nudus* (Dana, 1851). Crabs represent

ideal animals for such studies because a) they are readily maintained in the laboratory, b) they have a large number of easily measured, bilaterally symmetrical characters, c) they grow by molting in discrete steps with large size changes from one molt to the next (Hartnoll, 1982), and d) they produce molts that are sufficiently durable to yield a permanent record of external morphology throughout growth.

If subtle asymmetries are determined genetically, or are determined early in development, the sign and approximate magnitude of a character's right-minus-left difference should persist over time. If subtle asymmetries arise due to random processes acting continuously and independently throughout ontogeny or growth, either the sign or the magnitude (or both) should vary as the individual grows, particularly when individuals grow in discrete steps of large magnitude, as they do in most crustaceans.

Materials and methods

Collection and maintenance of crabs

Individual *Hemigrapsus nudus* were collected on May 24, 1988 from a cobble beach on the east side of Dixon Island (125°07'N, 48°51'W), near Bamfield, British Columbia, Canada, where they occur intertidally in great numbers. To minimize potential problems associated with sexual dimorphism and size-scaling of metrical characters, only female crabs between 9 and 13 mm carapace width were collected. Individual crabs were isolated in plastic freezer containers with plastic mesh sides that were held partially immersed in running seawater at the Bamfield Marine Station for approximately 14 months (from late May 1988 to mid-August 1989). During this time, animals were held under ambient laboratory conditions (natural photoperiod, temperatures ranging from 7.3-11.3 °C) and were fed *ad libitum* on a diet of various foods obtained fresh from the original collection site (e.g. *Ulva* and other seaweeds, mussels, and barnacle encrusted rocks, from which they scrape encrusting diatoms) and pieces of fish. This laboratory environment was designed to minimize stresses crabs normally experience in the intertidal zone, including salinity and temperature fluctuations, aggressive interactions, and foraging on rugged substrata.

Twice weekly, cages were cleaned and any molts were removed for measurement.

Measurement protocol

Thirty females were collected originally, but only ten were measured completely because: 1) several crabs died, 2) exoskeletons were incomplete on some individuals, and 3) the large numbers of measurements required per molt (28×2 characters each measured five times) made the measurement of more individuals impractical.

A total of 28 limb segment lengths, and one correlate of general body size (carapace width) were measured on each molt. The proximo-distal length of four limb segments, the merus (three different measurements on all but the clawed limb), carpus, propus, and dactylus, was measured on each of the five pereopods. The three merus measurements were treated as independent traits in our analyses, since their asymmetry values were not significantly correlated: no indication of any association was detected when individuals were pooled and the characters were plotted against one another in pairs (e.g. merus a vs. merus b, merus b vs. merus c, and merus a vs. merus c) and least-squares regression was performed.

Subtle asymmetries in metrical characters that give rise to FA are small and random, and therefore indistinguishable from measurement error without replicate measurements (Palmer & Strobeck, 1986). Great care was taken to reduce the contribution of measurement error to our estimates of the between-sides variance. Limb segments were measured at approximately $80\times$ magnification using a dissecting microscope fitted with a camera lucida aligned over a 500 DPI resolution digitizing tablet linked to a Macintosh computer. Each intact limb was oriented so as to be viewed perpendicular to the plane of maximum traverse (defined by the proximo-distal and dorso-ventral axes). Individual segments were centered in the field of view and measured once prior to moving to the next segment on the same limb. Care was taken to orient characters so that both endpoints of linear measurements were in the plane of focus before measuring. This avoided error due to refocussing between points, which would alter the calibration of the graphics tablet, and also avoided parallax errors. To further reduce the impact of measurement error, this sequence of measurement was repeated independ-

ently five times (i.e. one set of measurements was completed on all limbs for a single molt before beginning a second set of measurements). Thus we obtained a very accurate estimate of measurement error that incorporated errors arising from a) aligning limbs on the measurement stage, b) relocating character endpoints, and c) random fluctuations of the electronic equipment. Carapace widths were also measured five times with the digitizing arrangement described above only at $40\times$ magnification.

Test for preservation artifacts

Estimates of error due to preservation artifacts, such as distortion due to fixation or drying, were made by measuring a set of representative characters on two fresh individuals (seven characters on one and 13 on the other), which were baked for 24 h at 60-70 °C and remeasured. These two crabs were not part of the group of ten selected for complete measurement, although the characters measured were a subset of those used in the main analysis.

Measurement error and tests for departures from ideal FA

Tests for the significance of the between-sides variance relative to measurement error were conducted following the analysis of variance (ANOVA) procedure outlined in Palmer and Strobeck (1986). Before computing means and differences for each segment on a limb in all subsequent analyses, replicate measurements were averaged. Thus, each right-minus-left difference for a given limb segment was a difference between two averages, not an average of five differences.

FA is defined as a normal distribution of *R-L* differences about a mean of zero (VanValen, 1962). Because other patterns of statistical variation of *R-L* may render characters inappropriate for studies of developmental stability (Palmer & Strobeck, 1992) it was important to test for departures from ideal FA. Unfortunately, the small number of individual crabs measured in the present study (ten) greatly reduced our ability to detect departures from ideal FA (e.g. antisymmetry and directional asymmetry) in our samples. Further, since one of our goals was to determine whether subtle asymmetries varied at random during ontogeny, we could not assume *a priori* that they were random to en-

large our sample sizes to 30 by including the three molts for each individual crab. Fortunately, we had access to a larger data set collected by two students as part of a graduate course co-taught by one of us (A.R.P.) at the Friday Harbor Laboratories, Friday Harbor, Washington, in 1986. In this study of FA in *Hemigrapsus nudus*, relatively few (five) characters were measured three times on a large number (80) of individuals of both sexes collected from the field. The measurement procedure was similar to that outlined above, but data were collected using a lower resolution graphics tablet (200 DPI) attached to an Apple II+ computer. We analyzed the data for females only.

Tests for persistence of subtle asymmetries over successive molts

To test for the persistence of subtle asymmetries over successive molts, we treated each individual crab as a 'sample' of from 21 to 28 characters. The question posed was: do characters that exhibit a larger than average asymmetry in one molt of an individual crab exhibit a larger than average asymmetry in the subsequent molt? For each crab we regressed the departure from symmetry ($R-L$) in one molt against the departure from symmetry in the previous molt for all characters measured for that crab. Thus, for each crab we obtained two regressions: molt 2 vs molt 1 and molt 3 vs molt 2.

Data of this type do not conform to the assumptions of a least-squares linear regression analysis, where the X-axis variate is measured without error and all error variation is assumed to parallel the Y-axis (Sokal & Rohlf, 1981). In other words, in this analysis, there is no legitimate reason to define 'dependent' and 'independent' variables, nor is there any expectation for different error variances along the two axes. Both axes were $R-L$ differences, measured in identical fashion, and were of similar magnitude. To complicate matters further, the appropriate form of Model II regression for such data remains in dispute (LaBarbera, 1989). We thus estimated two alternate descriptors of the slope of our bivariate scattergrams: reduced-major-axis (RMA, Sokal & Rohlf, 1981, pp. 550) and the principal or major axis of a bivariate scattergram (MA). RMA slopes were obtained by computing a least-squares linear regression slope and dividing it by its correlation coefficient (LaBarbera, 1989).

MA slopes were obtained according to the procedure outlined in Sokal and Rohlf (1981, pp. 596). Thus for each molt interval, we obtained ten independent RMA slopes and ten independent MA slopes, one per crab.

To guard against the possibility that one individual crab exhibiting unusually large and persistent asymmetries would bias our conclusions about the persistence of asymmetries, we obtained an average overall slope by computing the arithmetic mean of the ten slopes obtained independently for each crab. In other words, we restricted our degrees of freedom in the final analysis by treating each regression for an individual crab as a single datum. For comparison, we also computed a single RMA and MA slope for the data from all crabs pooled for each molt interval. Finally, because correlations between molts were very low for some individuals, yielding slopes of low certainty, we re-computed the mean RMA and MA slope using only crabs exhibiting significant or nearly significant correlations between characters from molt to molt.

Paired-t tests, tests for skew and kurtosis of distributions of $R-L$, Mann-Whitney U Tests, analyses of variance and regression analyses were conducted with Statview II (Ver. 1.03, Abacus Concepts).

Results

Tests for preservation artifacts

The 20 characters measured to test for preservation artifacts shrank an average of $0.026 (\pm 0.0033, SE)$ mm when dried, corresponding to a shrinkage of $0.53\% (\pm 0.069)$. Differences between sides did not appear to be altered significantly by the harsh drying process. The average difference between sides (mean $|R-L|$) was 0.060 ± 0.0106 mm before drying and 0.064 ± 0.0101 mm after drying, and this change was not significant ($P = 0.070$, paired-T test). This test of preservation artifacts was liberally abusive: no crab used in the main analysis was exposed to such strong desiccation before measurement. No significant asymmetrical distortion due to baking and drying was observed, hence crab exoskeletons appeared durable enough to make preservation artifacts negligible relative to other sources of error.

Table 1. Proximo-distal length, and statistics describing frequency distribution shape of the differences between sides of limb segments of the posterior-most pair of walking legs in female shore crabs, *Hemigrapsus nudus*, from San Juan Is., Washington, USA.

Statistic	Limb segment			
	Merus	Carpus	Propus	Dactyl
<i>N</i>	40	40	40	40
a) $(R+L)/2$:				
Mean (SE)	6.97 (0.124)	3.91 (0.074)	2.76 (0.044)	3.45 (0.063)
b) $(R+L)$:				
Mean	0.028	-0.013	0.012	0.017
(SE, P^\diamond)	(0.0311, 0.37)	(0.0253, 0.61)	(0.0307, 0.70)	(0.0309, 0.59)
Kurtosis (P) [§]	0.804 (0.27)	-0.14 (0.85)	•2.082 (0.012)	1.384 (0.06)
Skew (P) [§]	0.45 (0.23)	-0.584 (0.12)	0.605 (0.11)	-0.271 (0.47)
c) $ R-L $:				
Mean (SE)	0.14 (0.022)	0.125 (0.016)	0.148 (0.019)	0.143 (0.021)
d) ANOVA*:				
Error MS	0.0185	0.0127	0.0167	0.0248
Interaction MS (P)	•0.058 (<0.001)	•0.038 (<0.001)	•0.057 (<0.001)	•0.059 (<0.001)
Sides MS (P)	0.0468 (0.37)	0.0106 (0.60)	0.008 (0.71)	0.0235 (0.59)
σ^2_i (approx. df)	0.013 (17.7)	0.009 (17.0)	0.013 (19.0)	0.011 (12.6)
e) FA5**	0.039	0.025	0.037	0.038
f) Size dependence of FA†				
Slope	-0.01	-0.04	0.03	0.07
(SE, P)	(0.029, 0.70)	(0.034, 0.22)	(0.072, 0.72)	(0.053, 0.19)

◇ P = probability that mean $(R-L)$ differs from zero.

§ SE values for skew and kurtosis are based on sample sizes (Sokal & Rohlf, 1981), and correspond to 0.374 and 0.733 for skew and kurtosis respectively for an N of 40.

* Analysis of variance procedure from Palmer and Strobeck (1986) for testing the significance of the between-side variance, directional asymmetry, and for partitioning the between-sides variance (σ^2_i) from the interaction MS. df- degrees of freedom.

** Index 5 of Palmer and Strobeck (1986): $\Sigma (R-L)^2/N$.

† Regression analysis of the dependence of character asymmetry, $|R-L|$, on character size, $(R+L)/2$.

• Indicates a statistically significant probability.

Measurement error, departures from ideal FA and effects of character size

For the preliminary sample of 40 female *Hemigrapsus nudus* from San Juan Island (Table 1d) the between-sides variance (Interaction MS) was significantly larger than measurement error for all four limb segments. Thus we were justified in analyzing patterns of variation in the between-sides variance in greater detail.

For this larger sample of crabs, all characters appeared to exhibit ideal FA (Palmer & Strobeck, 1992). None of the mean right-minus-left differences were significantly different from zero (Table

1b), thus there was no evidence for directional asymmetry. In addition, frequency distributions of right-minus-left differences exhibited no significant skew (Table 1b). One trait (propus length) exhibited significant kurtosis and a second (dactyl length) nearly significant kurtosis (Table 1b). In both cases kurtosis values were positive, signalling the presence of leptokurtosis (Sokal & Rohlf, 1981). There was no evidence of antisymmetry (platykurtosis) or other peculiar departures from ideal FA (Palmer & Strobeck, 1992).

The between-sides differences were also significantly larger than the measurement error for 28 characters measured on individual crabs in the pre-

Table 2. ANOVA test for significance of non-directional asymmetry (= interaction) relative to measurement error for 28 limb segments measurement five times on a single individual *Hemigrapsus nudus*, from Barkley Sound, to illustrate the relative magnitudes of measurement error and between-sides variation. Analyses for other individual crabs yielded similar results.

Source of variation	df	MS	F	P
Character	27	21.090	2343.3§	<0.0001
Sides	1	0.048	5.3§	0.030
Interaction	27	0.009†	237.5	<0.0001
Error	224	3.96×10^{-5}		

§ MS tested over Interaction MS, see Palmer and Strobeck (1986).

† the between-sides variance, $\sigma^2_i = (0.00941 - 3.96 \times 10^{-5})/5 = 0.00187$ (Palmer & Strobeck, 1986).

study (Table 2). Following the procedure outlined in Palmer and Strobeck (1986), the variance between sides (due to all non-directional asymmetries; σ^2_i of Palmer & Strobeck) was 0.00187. Thus the between-sides variance was 47.3 times greater than the variance among repeated measurements of the same limb segment (3.96×10^{-5}).

The smaller sample of crabs from which we obtained multiple molts also yielded no evidence of directional asymmetry. None of the 28 characters measured on the first or third molts ($N = 10$ per character) exhibited statistically significant directional asymmetry when subject to a sequential Bonferroni criterion which assures a table-wide probability of $P = 0.05$ (Rice, 1989; $P = 0.05/56$, data not shown). Furthermore, none of the six first-molt limb segments and only one of six third-molt limb segments (merus a) exhibited a barely significant

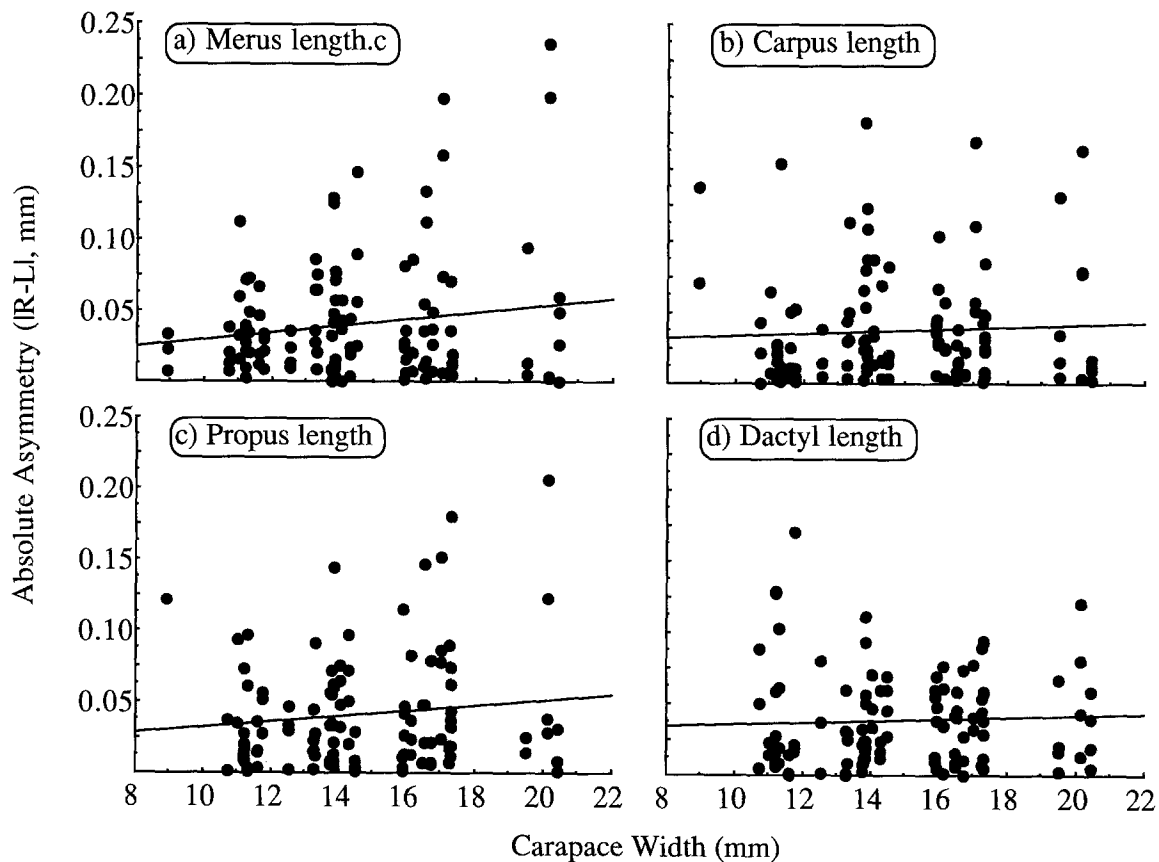


Fig. 1. Dependence of absolute asymmetry ($|R-L|$) on a measure of body size (carapace width) for four different limb segments on the walking legs of Bamfield *Hemigrapsus nudus*. Each graph presents pooled data for three molts of all four walking legs of ten individual crabs ($N = 120$). Each point corresponds to one limb segment on a single limb of a single molt. Least-square linear regression equations are: a) $Y = 0.0024 X + 0.0058$, $r^2 = 0.026$, $P = 0.086$; b) $Y = 0.0007 X + 0.0269$, $r^2 = 0.003$, $P = 0.59$; c) $Y = 0.0019 X + 0.0133$, $r^2 = 0.016$, $P = 0.19$; d) $Y = 0.0006 X + 0.0300$, $r^2 = 0.0022$, $P = 0.62$.

Table 3. Body size, average values for signed ($R - L$) and unsigned $|R-L|$ asymmetries, and percent asymmetry values $\{100 \times |R-L| / [(R + L)/2]\}$ for limb segments of the shore crab *Hemigrapsus nudus* from Bamfield, BC. Entries in section (a) were computed on between 21 and 28 different limb segments on individual molts. All values in mm. SE = standard error.

Crab #	Molt #	Carapace width	(R-L)		R-L			n
			mean	SE	mean	SE	% asym.	
a) Individual molts								
1	1	11.00	0.017	0.0102	0.041	0.0092	1.49%	21
	2	13.85	0.029	0.0110	0.048	0.0055	1.09%	28
	3	15.94	0.011	0.0084	0.034	0.0086	0.67%	24
2	1	11.20	-0.011	0.0064	0.025	0.0045	0.75%	28
	2	13.74	0.002	0.0060	0.022	0.0046	0.56%	27
	3	16.49	0.002	0.0061	0.022	0.0043	0.42%	24
3	1	11.20	-0.011	0.0072	0.026	0.0036	0.81%	28
	2	14.03	0.000	0.0091	0.041	0.0055	0.93%	28
	3	16.52	0.016	0.0117	0.047	0.0044	0.85%	27
4	1	11.75	-0.013	0.0092	0.034	0.0076	0.88%	26
	2	14.31	0.014	0.0070	0.029	0.0068	0.65%	28
	3	17.28	0.012	0.0073	0.027	0.0051	0.48%	26
5	1	11.33	-0.021	0.0094	0.036	0.0052	1.09%	27
	2	13.87	-0.013	0.0129	0.055	0.0070	1.22%	28
	3	16.76	0.007	0.0066	0.025	0.0083	0.46%	28
6	1	13.79	0.036	0.0166	0.059	0.0048	1.37%	27
	2	17.01	0.066*	0.0169	0.076	0.0135	1.62%	26
	3	20.13	0.055	0.0200	0.079	0.0129	1.34%	28
7	1	12.52	0.015	0.0057	0.025	0.0163	0.63%	26
	2	17.30	0.016	0.0094	0.040	0.0040	0.71%	28
	3	20.46	0.027	0.0113	0.039	0.0066	0.58%	28
8	1	11.63	0.004	0.0053	0.021	0.0100	0.59%	28
	2	14.49	0.000	0.0093	0.033	0.0032	0.76%	28
	3	17.33	0.002	0.0120	0.045	0.0068	0.78%	25
9	1	13.25	0.007	0.0093	0.038	0.0070	0.74%	28
	2	16.13	-0.008	0.0094	0.040	0.0071	0.75%	26
	3	19.50	-0.003	0.0131	0.045	0.0055	0.65%	26
10	1	10.73	0.018	0.0068	0.027	0.0104	0.78%	25
	2	13.34	0.010	0.0108	0.041	0.0051	1.03%	26
	3	15.93	0.014	0.0076	0.031	0.0066	0.59%	28
b) Average for each molt								
Molt 1 mean		11.6	0.002		0.033		0.88%	256
SE		0.08	0.0031		0.0023		0.065	
Molt 2 mean		14.5	0.010		0.042		0.92%	266
SE		0.11	0.0035		0.0024		0.052	
Molt 3 mean		17.4	0.016*		0.042		0.73%	262
SE		0.12	0.0037		0.0029		0.049	
c) All molts pooled								
Mean		14.5	0.010*		0.039		0.84%	790
SE		0.10	0.0020		0.0015		0.032	

* $0.01 < P < 0.05$ according to sequential Bonferroni criteria (Rice, 1989).

directional asymmetry ($P = 0.033$, standard Bonferroni criterion) when data were pooled across all walking legs ($N = 40$; data not shown). We are thus unable to eliminate completely the possibility that some directional asymmetry was present. However, if it was present, it was very subtle, since we were

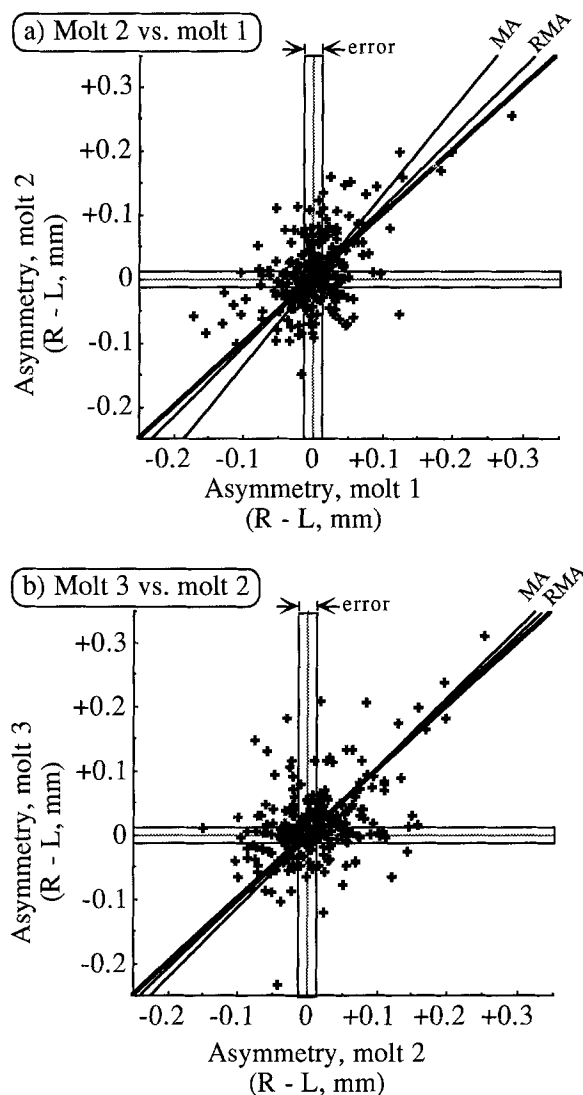


Fig. 2. Persistence of asymmetries in limb segments between successive molts in individual shore crabs *Hemigrapsus nudus*: a) molt 2 vs. molt 1, b) molt 3 vs. molt 2. Data were pooled across all limb segments for all crabs to compute MA and RMA slopes. The heavy solid line indicates perfect persistence of asymmetries. The finer lines indicate observed slopes. MA-major axis slope, RMA-reduced major axis slope. See Tables 4a and 4b for actual slopes. $N = 259$ and $N = 258$ in a and b respectively. Error-95% confidence intervals to illustrate the magnitude of measurement error ($\pm 2 \times \sqrt{\text{MS error from ANOVA}}$, Table 2).

only able to detect it in one of the 96 comparisons ($56 + 40$) when subject to sequential Bonferroni criteria.

Finally, we detected no association between the magnitude of asymmetry and character size, either among individuals for a particular character (Table 1f), or among leg segments (compare values in Table 1c or 1e to those in 1a) in the sample of 40 females from San Juan Island. Although limb segments differed in size by more than a factor of two, estimates of FA varied by only a factor of 0.2 (Table 1c) to 0.5 (Table 1e), depending on the index used. Hence we did not need to correct for size effects on FA (Palmer & Strobeck, 1986).

A similar analysis of each limb segment measured on three successive molts in Barkley Sound *H. nudus* also revealed no significant association between $|R-L|$ and carapace width when examined across all molts of all crabs (Fig. 1).

Change in overall asymmetry of successive molts

When averaged across all characters for a given molt, differences were observed among molts of individual crabs as well as among crabs (Table 3). One individual exhibited a significant right bias in molt 2 when averaged across all characters (crab #6). This crab also exhibited the highest variation of $R-L$ for all three molts; however, it also exhibited a significant right skew compared to other crabs.

The absolute magnitude of deviations from symmetry averaged 0.039 mm across all molts of all crabs (Table 3c). Expressed as a relative measure, the absolute asymmetry averaged over all molts was 0.84% of character size (Table 3c). As crab size increased from molt 1 to molt 2, average absolute asymmetry increased significantly from 0.033 to 0.042 mm ($P < 0.001$, Mann-Whitney U Test), but from molt 2 to molt 3 average absolute asymmetry remained unchanged (Table 3b). As a consequence, the level of relative asymmetry (scaled by character size) declined by nearly one quarter between molts 2 and 3, a decline that was also significant statistically ($P < 0.001$, Mann-Whitney U Test). Note that non-parametric tests are more appropriate for these comparisons because frequency distributions of $|R-L|$ are truncated normal, and hence seriously skewed to the right.

Table 4. Growth increments and three statistical descriptors of the associations between subtle asymmetries in successive molts of the Bamfield sample of laboratory-grown *Hemigrapsus nudus*. Growth increments were computed as the percentage increase in width of the carapace (difference between successive molts over smaller molt X 100). *a*) and *b*): association between (R-L, mm) in successive molts for 21 to 28 characters measured on individual crabs. *c*) Mean of associations between (R-L, mm) in successive molts computed using only crabs from *a* and *b* that exhibited a nearly significant LLR regression ($P < 0.1$; mean of values for crab # 1, 3, 4, 6, 7 and 8 from section *a* and crab # 3, 5, 6 and 10 from section *b*). *d*) As for *c* except means computed using only crabs that exhibited a significant LLR regression ($P < 0.05$; mean of values for crab # 3, 4, 6, 7 and 8 from section *a* and crab # 5, 6 and 10 from section *b*) LLR regression. N = number of characters per crab (or # characters X # crabs). LLR-least squares linear regression statistics (P indicates significance level of regression). RMA = statistics for reduced major axis (Sokal & Rohlf, 1981, pp. 550; P = indicates significance of departure from an expected slope of 1.0). MA = statistics for principal or major axis of a bivariate scattergram (Sokal & Rohlf, 1981, pp. 596; P = indicates significance of departure from an expected slope of 1.0). SE = standard error. r = correlation coefficient.

Crab #	Growth Incr.		LLR						RMA		MA		
	(%)	N	slope	SE	r	P	Ave.X	Ave.Y	Slope	P	Slope	P	
a) Molt 2 vs Molt 1													
1	25.0	21	0.49	0.253	0.40	0.070	0.017	0.032	1.21			1.567	
2	18.5	27	0.13	0.181	0.14	0.478	-0.012	0.002	0.92			0.555	
3	20.2	28	0.78	0.197	0.61	<0.001	-0.011	0.000	1.27			1.473	
4	21.8	26	0.35	0.131	0.48	0.014	-0.013	0.014	0.73			0.538	
5	18.3	27	0.42	0.253	0.32	0.108	-0.020	-0.017	1.33			2.280	
6	23.4	25	0.75	0.131	0.77	<0.001	0.038	0.068	0.98			0.971	
7	38.2	26	0.72	0.324	0.41	0.037	0.015	0.014	1.74			3.155	
8	24.6	28	1.27	0.236	0.73	<0.001	0.004	0.000	1.75			2.096	
9	21.7	26	0.26	0.196	0.26	0.193	0.005	-0.008	1.00			1.461	
10	24.3	25	-0.05	0.321	0.03	0.882	0.018	0.007	-1.54†			-28.42†	
Mean	23.6		0.51				0.004	0.012	1.21	0.110		1.57	0.081
SE	1.60		0.108				0.0051	0.0065	0.118			0.283	
Pooled		259	0.60	0.063	0.51	<0.001	0.003	0.010	1.17	0.008		1.35	0.014
b) Molt 3 vs Molt 2													
1	15.1	24	-0.04	0.159	0.05	0.807	0.023	0.011	-0.75†			-0.089†	
2	20.0	24	0.18	0.191	0.20	0.356	0.003	0.002	0.92			0.648	
3	17.8	27	0.42	0.241	0.33	0.095	0.001	0.016	1.28			1.996	
4	20.8	26	0.13	0.203	0.13	0.526	0.012	0.012	1.00			1.013	
5	21.0	28	0.20	0.094	0.39	0.042	-0.013	0.007	0.52			0.257	
6	18.4	26	0.97	0.156	0.78	<0.001	0.066	0.062	1.23			1.302	
7	18.3	28	0.32	0.229	0.26	0.175	0.016	0.027	1.21			1.969	
8	19.6	25	0.20	0.266	0.16	0.459	0.003	0.002	1.29			3.621	
9	20.9	24	0.48	0.285	0.34	0.107	-0.009	-0.001	1.42			2.518	
10	19.4	26	0.31	0.141	0.41	0.036	0.010	0.013	0.76			0.324	
Mean	19.1		0.32				0.011	0.015	1.07	0.500		1.52	0.202
SE	0.6		0.086				0.0070	0.0059	0.098			0.371	
Pooled		258	0.47	0.058	0.45	<0.001	0.011	0.015	1.04	0.536		1.07	0.606
c) Based on crabs where LLR Regression $P < 0.1$ only (N = 10 crabs)													
Mean	23.0		0.62				0.011	0.023	1.15	0.220		1.37	0.230
SE	1.9		0.105				0.0079	0.0076	0.129			0.286	
d) Based on crabs where LLR Regression $P < 0.05$ only (N = 8 crabs)													
Mean	23.4		0.67				0.012	0.022	1.12	0.476		1.26	0.472
SE	2.2		0.128				0.0099	0.0095	0.162			0.349	

† excluded from computation of means across crabs in a) and b) because of extremely low r.

Persistence of subtle asymmetries over successive molts

Subtle asymmetries detected in individual limb segments appeared to persist over successive molts for all ten crabs over both molt intervals (Fig. 2, Table 4). For each of the ten crabs examined, segments that exhibited a departure from symmetry in one molt tended to exhibit a departure in the same direction in the subsequent molt. In all, 18 out of 20 slopes for the molt to molt comparisons were positive (Table 4). The two slopes that were negative were also those that had the lowest correlation coefficients by nearly a factor of three. The probability of obtaining nine out of ten slopes positive due to chance, as observed both for molt 1-2 (Table 4a) and for molt 2-3 (Table 4b), would simply be the binomial probability $P = 0.002$.

In addition, the magnitude of the subtle asymmetries appeared to be approximately the same from molt to molt. The mean RMA and MA slopes for both molt intervals were significantly greater than zero, but neither was significantly different from 1.0 (Table 4a,b; two-tailed t-test). When data were pooled across all limb segments and all crabs, to estimate a single RMA and MA slope for each molt interval, both the RMA and MA slopes were significantly greater than 1.0 in the first molt interval (Table 4a) but were not significantly greater than 1.0 in the second molt interval (Table 4b). Finally, when mean slopes were computed using only data for crabs that exhibited nearly significant ($P < 0.1$) or significant ($P < 0.05$) statistical associations from molt to molt based on LLR regressions, neither the mean RMA nor MA slopes differed significantly from 1.0 (Table 4c,d).

Discussion

Confirmation of fluctuating asymmetry

Before measures of the between-sides variance [e.g. $\text{Var}(R-L)$ or mean $|R + L|$] can be interpreted as measures of developmental stability, the between-sides variation must be shown a) to be larger than the measurement error (Palmer & Strobeck, 1986) and b) to conform to ideal FA (Palmer & Strobeck, 1992). For a distribution of $R-L$, ideal FA corresponds to a mean of zero with normally

distributed variation (VanValen, 1962). For both sets of data we examined, the larger preliminary sample of *Hemigrapsus nudus* from the San Juan Islands (Table 1) and the smaller sample of crabs from Barkley Sound (Table 2), we confirmed that the subtle departures from symmetry that give rise to FA were significantly larger than our measurement error. In addition, these tests also revealed no statistically significant directional asymmetry (Tables 1 & 2), or antisymmetry (platykurtosis) or skew (Table 1). Three of the four limb segments for which we had adequate sample sizes exhibited leptokurtosis and one exhibited platykurtosis (Table 1) however, only one of these kurtoses was statistically significant (leptokurtosis for the propus, Table 1). As discussed elsewhere (Palmer & Strobeck, 1992), the biological significance of departures from normality in the direction of leptokurtosis is unclear, and we attribute no biological significance to it here.

When examining our pooled data, we did detect hints of a right-bias to departures from symmetry, but these became apparent only when pooling measurements together, either at the level of individual crabs (Table 3a), at the level of molts (Table 3b), or pooling the data for all molts for all crabs (Table 3c). These P values, however, need to be interpreted with great care for two reasons. First, when pooled across multiple characters for an individual crab, as in Table 3a, a significant departure of mean $(R-L)$ indicates that more *characters* exhibited a side-bias than would be expected due to chance. In other words, it does not necessarily mean any individual characters exhibited directional asymmetry, it only means that an individual crab exhibiting a side-bias for one character was more likely to exhibit a side-bias in the same direction for another character. Second, when pooling across all crabs (Table 3b), or across all molts of all crabs (Table 3c), a significant bias could arise because of a lack of independence. If, by chance, six of the ten crabs we studied exhibited a slight right bias correlated across several characters, then pooling a large number of measurements based upon this small number of crabs would give the appearance of a statistically significant right bias to the entire sample. Finally, even when all data were pooled, the observed directional asymmetry was only 0.010 mm which was roughly one-quarter the magnitude of the average absolute difference be-

tween sides (0.039 mm, Table 3c). An average right-bias of this small a magnitude is very unlikely to have given rise to any of the patterns we observed between molts. Thus, although we can not reject entirely the possibility that some slight directional asymmetries were present, the data least subject to analytical bias (Table 1) did not reveal any, and we suspect the apparent directional asymmetries revealed in the pooled data (Table 3) were statistical artifacts of pooling.

In summary, we conclude that the departures from ideal FA we did observe were not only small in magnitude relative to the patterns of between-molt variation we were studying, but appeared to have arisen as a statistical byproduct of pooling data or of conducting a large number of tests. Thus for the purpose of studying between-molt variation, we felt confident that all characters exhibited ideal FA. The presence of a persistent, and significant right-bias (in one of three molts) in a single individual (Crab #6, Table 3) when all characters were pooled suggests the tantalizing possibility that the symmetry of some individual crabs may be disrupted very early in development, leading to a subtle, body-wide asymmetry. Additional study will be required to determine if body-wide asymmetries are more widespread.

Potential biases in the analyses of persistent subtle asymmetries

Another potential concern that arises in the sort of sampling scheme employed here is the independence of characters. If certain characters display directional asymmetry or antisymmetry of sufficient magnitude, they may exert a significant leverage on the slope of regressions of $R-L$ between molts. For example, if claw measurements were included as independent data points in an estimate of overall FA for an individual crab, but claws displayed antisymmetry, they might 'lever' the regression towards a positive slope, even though all other limbs exhibited FA.

To test for possible biases, we repeated our analyses on subsets of the data with certain groups of characters excluded. In one set of analyses, we excluded characters grouped by limb (e.g. all cheliped measurements, or all measurements of walking limb 1, etc.). In a second set, we excluded groups of serially homologous characters (e.g. all measure-

ments of the dactyl on all limbs, or all measurements of the propus on all limbs, etc.). For all 20 of these alternate analyses, the average slope of the least squares regression was not altered qualitatively (i.e. the sign was the same and the slope was similar). Thus neither particular limbs, nor particular limb segments appeared to exert a significant leverage in our analyses.

In addition, we conducted our analyses of persistent subtle asymmetries in such a way that a bias due to one crab would not exert an undue influence on the overall pattern. Hence, although one crab exhibited an overall right bias across all characters (Crab #6, Table 3), we computed an average of the nine individual RMA and MA slopes obtained from single crabs. This procedure yielded similar slopes to those obtained when all data were pooled to compute a single slope.

Our conclusion that subtle asymmetries persist over successive molts does not appear to result from biases in our data or analyses.

Measuring the persistence of subtle asymmetries during growth

FA is normally measured for a sample of individuals at a particular time or place, or under a particular set of conditions in the laboratory (Zakharov, 1992). It is thus a statistical descriptor of a sample, and not normally considered as an attribute of an individual organism. Some individuals may be more developmentally stable than others, and thus exhibit smaller departures from symmetry for a suite of characters. To obtain a multivariate measure of developmental stability, one would have to follow a procedure similar to that proposed by Zhivotovsky (1992). Studies of the origin of FA during development and the dynamics of its change in development and growth are conspicuously lacking. These considerations are of particular importance to the application of size scaling as well as to the use of FA for monitoring the condition of natural populations.

These questions have probably been overlooked for obvious methodological reasons. Because 'individuals' do not exhibit FA that can be quantified, except in cases where clone-mates or sub-components of modular organisms can be used to assess the asymmetry variance for a genotype (Palmer & Strobeck, 1986) or where individuals replace struc-

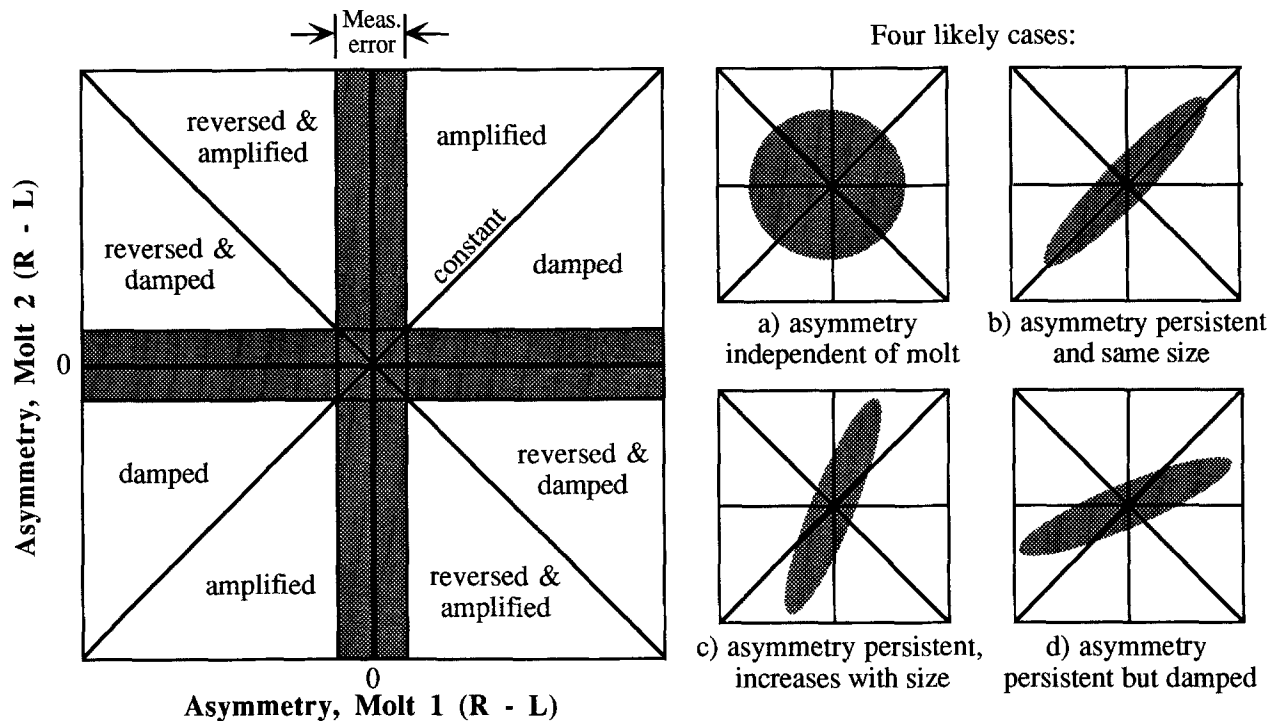


Fig. 3. Key for interpreting associations of subtle asymmetries between molts. Plots of $R-L$ differences from two successive growth stages will reveal whether departures from symmetry persist, and whether they are amplified or damped. Many patterns of association are possible, but four seem more likely than others: a) subtle asymmetries are independent from molt to molt (random determination; this pattern would also obtain if the between-sides variation was due exclusively to measurement error), b) subtle asymmetries persist at the same magnitude from one molt to the next (constant asymmetries), c) subtle asymmetries persist but are amplified in subsequent molts because characters are larger (size-dependent asymmetries), and d) subtle asymmetries persist but are damped in subsequent molts (damped asymmetries).

tures repeatedly throughout their life (e.g. bird feathers that are molted; Møller & Höglund, 1992), it is difficult to extract information about the developmental origins of subtle asymmetries for individuals from typical sampling schemes employed to study FA. The method for tracking the progress of asymmetries we have proposed here is limited primarily to laboratory growth studies because data must be collected from the same individual at different times during growth. Given this limitation, however, the method is quite easily applied to a wide variety of organisms. We chose crabs because they grow in discrete steps with easily preserved molts. Skeletal variation in vertebrates could also be measured throughout growth rather easily by using measurements from X-ray plates.

One encouraging implication of our study is that subtle asymmetries in metrical characters in crabs appeared to arise early in development and persist at a more or less constant magnitude throughout

growth. In this fashion, they are comparable to meristic characters that often appear to be fixed early in development (Taning, 1952; Wooten & Smith, 1986; but see Strawn, 1961, and Angus & Schultz, 1983, for evidence to the contrary). In addition, because the subtle asymmetries we observed did not increase significantly with character size, FA estimates from pooled samples containing a size range of crustaceans may not be seriously confounded by size-dependent variation (Palmer & Strobeck, 1986).

Interpreting persistent subtle asymmetries

Where subtle asymmetries may be followed over time, as in our study, we may ask how persistent such asymmetries are from one time interval to the next. Although many possibilities exist (Fig. 3), four seem more likely than others. First, departures from symmetry at one time may be completely

independent of those at a later time (Fig. 3a). In other words, subtle departures from symmetry could vary continuously and independently throughout growth. This pattern might arise in crustaceans, for example, where body size increments are large and occur in discrete increments at molting. The exoskeleton is shed completely, and the animals inflate dramatically with fluids before the new cuticle hardens (Hartnoll, 1982). This pattern would also arise, of course, where apparent subtle asymmetries were simply the result of measurement error, which further emphasizes the importance of separating measurement error from estimates of FA for metrical characters. Second, subtle departures from symmetry may become fixed early in development and then persist in the same direction and magnitude throughout subsequent growth (e.g. asymmetry is independent of character size; Fig. 3b). Third, subtle departures from symmetry may be fixed early in development, but increase in magnitude throughout subsequent growth as characters become larger (e.g. asymmetry is a constant proportion of character size; Fig. 3c). Fourth, subtle departures from symmetry that arise at any time during development may be damped or corrected during growth (Fig. 3d).

We should also point out here that care must be taken when computing slopes of model II regression where correlations of asymmetries between growth stages are very low. As the correlation coefficient goes to zero, the expected value of the RMA slope approaches 1.0, because the RMA slope is merely the ratio of the standard deviations of Y and X (Sokal and Rohlf 1981, pp. 550). The MA slope, on the other hand, will become very sensitive to the location of individual data lying just outside the bivariate distribution (e.g. see individual 10, Table 4a). We avoided these problems by repeating analyses using only cases where the correlations were significant or nearly so (Table 4c,d).

Persistence of subtle asymmetries in Hemigrapsus nudus: Relevance to developmental stability

Our conclusions are based on a small sample of crabs and therefore somewhat limited. However, the small departures from bilateral symmetry exhibited by *Hemigrapsus nudus* were, on average, preserved in both direction and magnitude in spite of large increases in overall body size accom-

plished in rapid leaps of growth. These patterns of molt to molt variation were thus most similar to case (b) of Fig. 3. Even though our measurement error was quite small (Fig. 2), these patterns were also unlike anything that could have been generated by measurement error, where a random scatter of points would be expected in the molt vs. molt plots (as in Fig. 3a).

We offer two hypotheses to account for the persistence of these subtle (<1% of character size) asymmetries through growth. First, individual crabs may have a genetic predisposition towards asymmetry that manifests itself as a continuous distribution of *R-L* differences when viewed at the level of a *sample* of individuals, but appears as persistent asymmetries over successive molts when viewed at the level of an individual. Second, developmental errors may have been fixed at some earlier stage of growth, and simply persisted throughout subsequent growth. At present we do not prefer one hypothesis over the other, and our data do not allow us to distinguish between them.

The first hypothesis is very difficult to assess since the published patterns yield contradictory conclusions. Attempts to select for a bias towards one side in characters that initially exhibited FA have been repeatedly unsuccessful in *Drosophila* (Maynard Smith & Sondhi, 1960; Coyne, 1987; Tuinstra, Dejong & Scharloo, 1990). These studies suggest that deviations from symmetry do not have a heritable basis. Nonetheless, strongly asymmetrical structures have evolved repeatedly in otherwise symmetrical organisms (Ludwig, 1932; Neville, 1976), suggesting that heritable variation for subtle deviations from symmetry must have existed at some point. In addition, (Palmer, Strobeck & Chipindale, 1993) have also suggested that certain patterns of normally-distributed variation in *R-L* may signal the presence of heritable variation.

The second hypothesis implies that a kind of morphological 'templating' may be at work: asymmetries tend to carry over from one molt to the next because the hydrostatic expansion of parts between molts preserves small irregularities under the stable conditions of the laboratory environment. We took individuals from the wild, where they experienced harsh and unstable conditions, and placed them in a controlled environment that interfered little with subsequent growth. It is puzzling, however, that if such 'templating' is in operation, the asymmetries

do not scale significantly with overall body size. All observed RMA and MA slopes were greater than 1.0, but in only one instance were they significantly so (Table 4a). The average percent increase in carapace width between molts was approximately 20%, thus if subtle asymmetries increased isometrically with body size they should have increased by roughly 20% (i.e. exhibited a slope of 1.2 between successive molts). In most cases, however, the residual variation in our data was sufficiently large that we could not distinguish between a slope of 1.0 and one of 1.2. Thus we cannot say with certainty that these subtle asymmetries did not scale with overall body size.

Perhaps most puzzling is that *Hemigrapsus nudus*, a species known to autotomize and regenerate entire limbs, seemed unable to correct for asymmetries presumably reflecting developmental noise (see expected pattern in Fig. 3d). A crab can regenerate an entire limb after two or three molts (McVean, 1982), yet subtle asymmetries persisted through two successive molts. Perhaps there is a threshold for asymmetry, above which correction can occur, and below which asymmetry persists through growth. Some exciting possibilities for future research arise from this supposition: Do such subtle asymmetries reproduce themselves in regenerated limbs? How do the other limbs compensate for the loss of a limb?

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