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Production of different phenotypes from the same genotype in the same environment by developmental variation

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SUMMARY

The phenotype of an organism is determined by the genes, the environment and stochastic developmental events. Although recognized as a basic biological principle influencing life history, susceptibility to diseases, and probably evolution, developmental variation (DV) has been only poorly investigated due to the lack of a suitable model organism. This obstacle could be overcome by using the recently detected, robust and highly fecund parthenogenetic marbled crayfish as an experimental animal. Batch-mates of this clonal crayfish, which were shown to be isogenic by analysis of nuclear microsatellite loci, exhibited surprisingly broad ranges of variation in coloration, growth, life-span, reproduction, behaviour and number of sense organs, even when reared under identical conditions. Maximal variation was observed for the marmorated coloration, the pattern of which was unique in each of the several hundred individuals examined. Variation among identically raised batch-mates was also found with respect to fluctuating asymmetry, a traditional indicator of the epigenetic part of the phenotype, and global DNA methylation, an overall molecular marker of an animal's epigenetic state. Developmental variation was produced in all life stages, probably by reaction–diffusion-like patterning mechanisms in early development and non-linear, self-reinforcing circuitries involving behaviour and metabolism in later stages. Our data indicate that, despite being raised in the same environment, individual genotypes can map to numerous phenotypes *via* DV, thus generating variability among clone-mates and individuality in a parthenogenetic species. Our results further show that DV, an apparently ubiquitous phenomenon in animals and plants, can introduce components of randomness into life histories, modifying individual fitness and population dynamics. Possible perspectives of DV for evolutionary biology are discussed.

Key words: genotype, phenotype, variation, marbled crayfish, development, growth, colour, reproduction, behaviour, sense organs.

INTRODUCTION

Variation of the phenotype is a central issue in biology because it is the basis for individuality, adaptation of populations to environmental fluctuations, and the evolution of biodiversity (Crawford and Oleksiak, 2007; Dow, 2007; Hallgrímsson and Hall, 2005; Pigliucci et al., 2006; West-Eberhard, 2003). Phenotypic variation can be produced by genetic differences, environmental influences and stochastic developmental events. Whilst the genetic and environmental components are rather well investigated, developmental variation (DV), also called 'intangible variation' or 'developmental noise', remained a largely untouched field of research (Astauroff, 1930; Falconer and Mackay, 1996; Gärtner, 1990; Peaston and Whitelaw, 2006; Veitia, 2005).

With the exception of fluctuating asymmetry, the right–left difference of a trait (Dongen, 2006; Møller, 2006; Nijhout and Davidowitz, 2003), DV has rarely been directly investigated, mainly because suitable laboratory models are not at hand (Peaston and Whitelaw, 2006). However, population genetic calculations (Falconer and Mackay, 1996) and some studies with parthenogenetic aphids and daphnids (Lajus and Alekseev, 2004; Warren, 1902), polyembryonic armadillos (Loughry and McDonough, 2002), human monozygotic twins (Falconer and Mackay, 1996), inbred lines of *Drosophila*, guinea pigs and mice (Astauroff, 1930; Gärtner, 1990; Peaston and Whitelaw, 2006; Wright and Chase, 1936), and cloned mammals and plants (Archer et al., 2003a; Archer et al., 2003b; Jaenisch and Bird, 2003; Scowcroft, 1985) revealed that DV often accounts for more than half of the total variation of a trait. Moreover, DV was assumed to contribute significantly to differences in aging and carcinogenesis of humans (Aranda-Anzaldo and Dent, 2003; Kirkwood et al., 2005) and to severely hamper standardization of test animals (Gärtner, 1990).

An ideal laboratory model for investigation of DV should produce high numbers of isogenic progeny, possess a variety of morphological traits that are easy to analyse, and enable rearing in simple environments. The Marmorkrebs or marbled crayfish, a varico-coloured parthenogenetic all-female species detected by us a few years ago (Scholtz et al., 2003), fulfils all of these requirements, and has further advantages such as easy accessibility to all life stages, adaptability to a wide spectrum of experimental conditions, indeterminate growth, direct development, stereotyped cell lineage in early development, broad behavioural repertoire and, most importantly, stepwise alteration of the phenotype by moulting (Alwes and Scholtz, 2006; Dohle et al., 2004; Seitz et al., 2005; Vogt and Tolley, 2004; Vogt et al., 2004). Moreover, the exuviae

produced by moulting yield permanent records of the morphological traits, permitting accurate analysis under the microscope and comparison of character states of individual crayfish throughout life.

The marbled crayfish, which appeared first in the mid 1990s in the German aquarium trade, is apparently a parthenogenetic strain of the North American cambarid *Procambarus alleni* (Faxon 1884), as revealed by analysis of the 16S hypervariable region of the mitochondrial genome (Keith Crandall, personal communication). Under optimal conditions it has a generation time of 5–6 months and clutch sizes of 50–400 eggs, depending on age. The eggs and the first two lecithotrophic juvenile stages are permanently carried under the mother's abdomen and are thus exposed to the same environment. Stage-3 juveniles, the first feeding stage, and also sometimes stages 4 and 5, adhere to the maternal pleopods to rest but leave them regularly for feeding. The late embryonic stages and first juvenile stages can be raised in very simple *in vitro* systems, facilitating standardization of the environmental conditions. Moreover, all live stages can be fed with the same pellet food as sole food source.

In the present study we have investigated DV in many hundred marbled crayfish of all ages with respect to development and growth, life-span, reproduction, coloration, number of olfactory and gustatory sense organs, behaviour, and the epigenetic markers fluctuating asymmetry and global DNA methylation. The emphasis of our experiments was on the analysis of variation within batches, which were shown to be genetically identical in the marbled crayfish (Martin et al., 2007), the batch-mates being exposed at any time to the same environmental and nutritional conditions. Food was generally given in excess. It was the aim of our experiments to test the suitability of the marbled crayfish for investigation of DV and to provide baseline data for future research on the relationship of epigenetics and phenotype and on environmental epigenomics.

MATERIALS AND METHODS

Animals and general measures

All experiments were performed with marbled crayfish [parthenogenetic strain of *Procambarus alleni* (Faxon 1884) according to Keith Crandall (personal communication)] from the laboratory population of G.V., which was founded in February 2003 from a single individual. Maintenance of the crayfish and experiments followed the animal care guidelines of the University of Greifswald.

To exclude non-DV sources of variation we took the following measures in addition to the use of a genetically uniform test species: (1) analysis of batches to minimize potential influences of mutations and seasonal rhythms; (2) rearing of animals under identical laboratory conditions to standardize macro-environmental parameters; (3) use of simplified rearing systems to minimize micro-environmental influences; (4) feeding of all life stages with the same pellet food as sole food source in excess, to exclude any influences of difference in food quality; (5) focussing on juvenile stages 1–6, as these stages can be determined reliably and are particularly peaceful; (6) regular inspection of the stock population for crayfish diseases (Vogt, 1999); (7) performance of the experiments and collection of all morphological and life history data by the same experienced person (G.V.). Responsibilities: G.V., idea, experiments, morphological and life history analyses; M.H. and C.D.S., analysis of microsatellites; M.T. and O.J.S., analysis of DNA methylation; and G.v.d.B., statistical analysis of meristic and metric traits.

Genetic analyses

To verify clonality of our experimental animals we chose the highly sensitive microsatellite technique. The following specimens were analysed: (1) the founder females A and B of the two laboratory lineages used for the experiments, (2) nine offspring of dam A from the same batch, (3) seven specimens of the oldest known German aquarium lineage established in 1995, and (4) three siblings of another German aquarium population established in 1998. For comparison, we analysed an aquarium-reared batch of the sexually reproducing *Procambarus clarkii*, which is closely related to the Marmorkrebs (Scholtz et al., 2003).

Genomic DNA was obtained from ethanol-preserved tissue from walking legs or the pleon, using the Puregene Kit (Gentra Systems, Minneapolis, MN, USA). PCR were performed in a final volume of 20 μl with annealing temperatures varying between 46°C and 58°C and 30 cycles. Successfully amplified products were ethanol-precipitated and cycle-sequenced in the automated sequencer ABI Prism 310 (Applied Biosystems, Darmstadt, Germany). The sequences obtained were then aligned and compared with the corresponding published sequences of *Procambarus clarkii* from GenBank prior to the use of 5′-labeled primers for fragment length analysis.

Ten microsatellite primer combinations, nine designed for the crayfish *Procambarus clarkii* (PclG-03, PclG-04, PclG-07, PclG-08, PclG-15, PclG-26, PclG-27, PclG-32, PclG-37) (Belfiore and May, 2000) and one for *Orconectes placidus* (locus 2.6) (Walker et al., 2002), were tested for potential use with the marbled crayfish. Out of these, two primer combinations rendered successfully amplified DNA with microsatellites in the marbled crayfish: PclG-04 and PclG-26. The primers used were TAT ATC AGT CAA TCT GTC CAG (forward) and TCA GTA AGT AGA TTG ATA GAA GG (reverse) for PclG-04 and CTG TAG GCC TTC ATG GAC TTC TTG (forward) and TGT TCA CAT CAG CAG GAG ATA ACT A (reverse) for PclG-26. The latter primers were newly designed by us, based on the sequences of *Procambarus clarkii* and the marbled crayfish.

Investigation of life history parameters, coloration, and morphological traits

Development and growth, life-span, reproduction, coloration, and morphological characters were investigated in several batches and monitored for a maximum of 910 days. Variation of development and growth was investigated in batches from dam A, dam B, daughters B_1, B_3, B_4 and B_5 and granddaughter $B_{3\text{-}1}$ in different environments, e.g. maternal pleopods, 12-well micro-plates, 18×6 cm net culture systems and 30×20 cm and 60×30 cm aquaria. The aquaria were equipped with a thin layer of gravel and stones as shelter. Feeding was started when all juveniles of a batch had moulted into stage 3. The only food source for all life stages was TetraWafer Mix, which was fed once a day *ad libitum*. The water was completely changed weekly, and the aquaria were carefully cleaned at these occasions. Excess food was siphoned out daily. In the smaller culture vessels water was replaced as necessary. The water source (tapwater) was the same for all experiments, as were temperature (room temperature) and light conditions (natural light rhythm) at a given time.

Long-term variation of growth and reproduction was studied in eight offspring of dam B (B_1–B_8), that were communally reared in a 60×30 cm aquarium until death, in 20 offspring of dam $B_{3\text{-}1}$ (groups C and D) that were communally reared in 30×20 cm aquaria for 365 days, and four individually raised offspring from dam $B_{3\text{-}1}$ (F_1–F_4) that were reared in 30×20 cm aquaria for 350

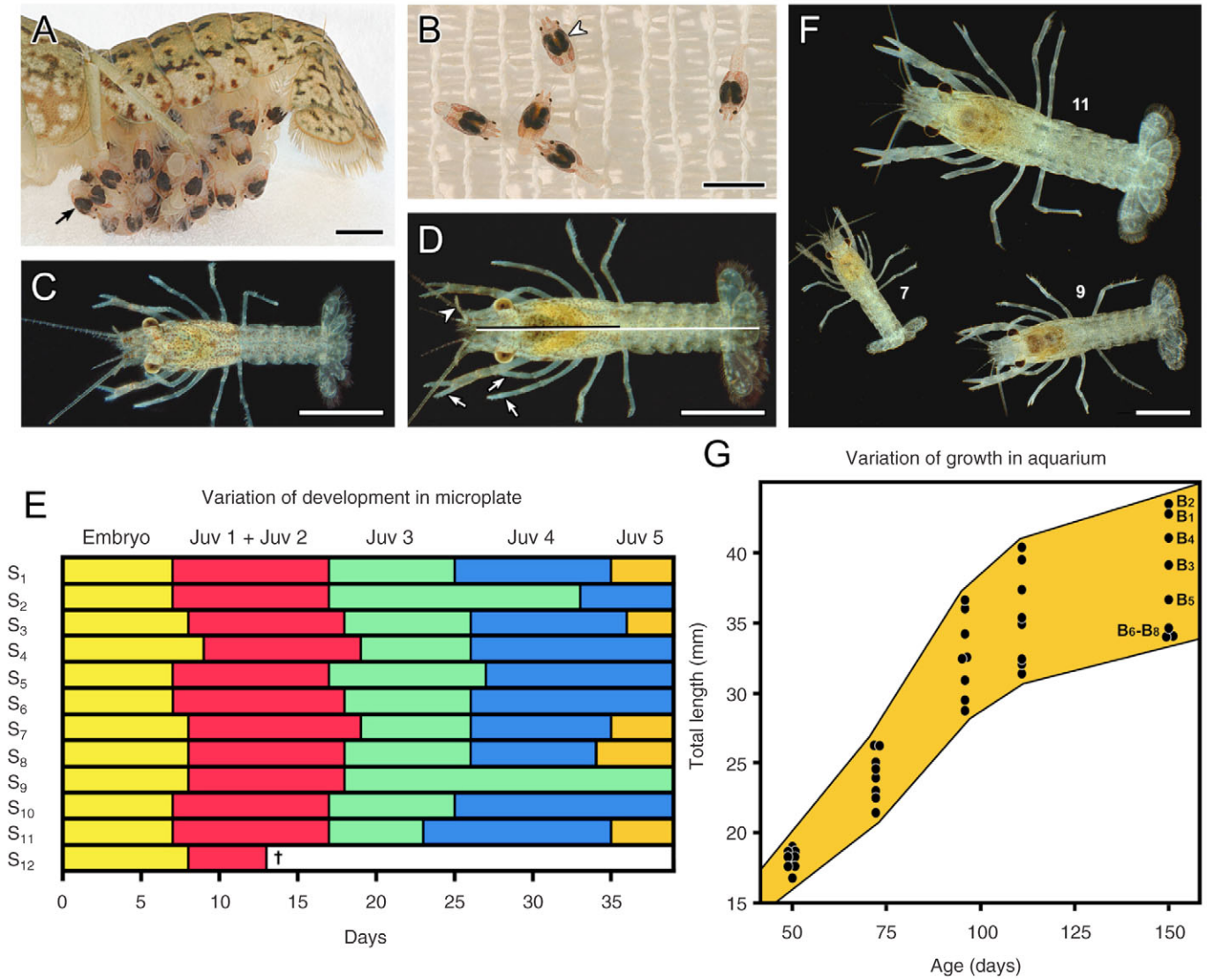


Fig. 1. Variation of development and growth in juveniles and adolescents. (A,B) Rather synchronous development of lecithotrophic stage-2 juveniles (arrow) from dam A on the maternal pleopods (A) and in net culture (B). Arrowhead in B denotes yolk reserves. Scale bars, 3 mm. (C,D) Size differences among feeding batch-mates removed from brooding dam A at day 35 after hatching: the juvenile in C is in stage 3 whilst its sib in D is already in stage 5. Markers in D denote traits and organs investigated in juveniles: carapace length (black line), total length (white line), outer branch of 1st antenna (arrowhead) bearing the olfactory aesthetascs, and chelae of pereiopods 1, 2 and 3 (arrows) bearing the gustatory corrugated setae. Scale bars, 2 mm. (E) Development of batch-mates (S_1–S_{12}) from dam B_5 raised individually in a 12-well microplate from late embryogenesis to juvenile stage 5 (Juv 5). Development is rather uniform in embryos and non-feeding stages 1 and 2 but becomes heterogeneous after onset of feeding in stage 3. (F) Growth differences of five juveniles from dam B that were size-matched in stage 6 and then cultured in an aquarium without shelter. 34 days later, one specimen was in stage 11, one in stage 9 and three were in stage 7. Scale bar, 4 mm. (G) Temporal development of size variation in eight adolescent batch-mates (B_1–B_8) from dam B reared communally under aquarium conditions. Specimens are numbered according to sequence of egg-laying, which started in B_1 at day 158 after hatching.

days. In these experiments temperature fluctuated between 18°C and 23.5°C but was the same for each group member at a given time. Further details of the various growth experiments are found in the respective paragraphs of the Results.

Variation of the colour pattern of the integument were investigated in 20 juveniles of dams A and B_1 and 10 adults (dams A, B and B_1–B_8). In the juvenile stages 1–7 we have analyzed the areola, a dorsal area of the cephalothorax (Fig. 3A), under the dissection microscope with respect to pattern similarity among batch-mates and individual pattern development. In adults we have examined the posterio-lateral areas of the carapace (Fig. 3B), using a digital camera with macro-lens, with regard to similarities among batch-mates, similarities between mother and offspring, and alteration of the marmoration motifs with time. Individual development of the colour pattern was monitored for a maximum of 880 days.

Variation of carapace length and numbers of olfactory and gustatory sense organs were investigated in progeny of dams A, B, B_1 and B_3, which were removed from the maternal pleopods in early stage 2 and then reared until stage 6 in 11×8 cm net culture systems. Stocking density was six specimens per vessel and water temperature was 20°C. Carapace lengths (tip of rostrum to posterior margin of carapace) (Fig. 1D) were measured using an eyepiece micrometer to the nearest 0.06 mm in specimens fixed in 70% ethanol. The olfactory aesthetascs and the gustatory corrugated setae were counted under the light microscope, using ethanol-fixed 1st antennae and pereiopods 1, 2 and 3 (Fig. 1D), respectively.

Specimens with regenerated or damaged appendages were excluded. Scanning electron micrographs of the aesthetascs and corrugated setae were obtained by routine methods (Vogt et al., 2004).

The differences in the means of carapace length (CL) and numbers of aesthetascs (Ae) and corrugated setae (CS) among batches and among juvenile stages were analysed by a bifactorial analysis of variance (ANOVA) with interaction, and differences in variation of the same parameter among batches and juvenile stages by Bartlett tests. Both methods rely on approximate normality of the residuals.

Modular relationships between traits were analysed in the same specimens by comparison of the coefficients of relative variation of CL and numbers of Ae and CL based on confidence intervals. We selected a relative measure of variation to ensure comparability among different juvenile stages. In order to provide precise confidence intervals, we used the geometric standard deviation $s_g=\exp\{\mathrm{sd}[\log(x)]\}$ as a measure of relative variation rather than the more traditional coefficient of variation $\mathrm{CV}=\mathrm{sd}(x)/\mathrm{mean}(x)$. For small relative variations – as in our case – both are approximately related as $s_g=1+\mathrm{CV}$. Confidence limits for s_g can be computed easily based on the χ^2-confidence limits for the estimated standard deviation $\mathrm{sd}[\log(x)]$. Again, the method relies on approximate normality. The statistical computations were done with the statistics software R (R Development Core Team, 2007).

Investigation of fluctuating asymmetry

Fluctuating asymmetry (FA), the deviation from perfect symmetry to the left (L) or right (R), was individually determined for the number of corrugated setae on pereiopods 1–3 and the number of aesthetascs. FAs were calculated according to the formula $100\times(\mathrm{R}-\mathrm{L})/(\mathrm{R}+\mathrm{L})\times\frac{1}{2}$ for 168 stage-2 to stage-6 juveniles from dams A, B, B_1 and B_3. Longitudinal development of FA was investigated by using the exuviae of offspring of dam B reared individually over a maximum of eight moulting stages.

Investigation of DNA methylation

Methylation of the DNA was measured in the hepatopancreas and abdominal musculature of four communally reared adolescent batch-mates and three communally reared adult batch-mates. Analyses were performed with capillary electrophoresis, using a novel and highly sensitive sample preparation technique (Schiewek et al., 2007). Each sample was measured at least 20 times.

Genomic DNA was isolated from frozen samples of the hepatopancreas and abdominal musculature with Qiagen Genomic-tip 20/G according to the supplier's instructions (Qiagen, Hilden, Germany). Only the last eluation step was changed: destilled water was used instead of TE-buffer. For hydrolysis and derivatization 1 μg DNA was diluted in 5 μl water and hydrolyzed by incubation for 3 h at 37°C with 4.2 μl of an enzyme mixture composed of micrococcal nuclease (MN) (150 mU μl^{-1})/spleen phosphodiesterase (SPD) (12.5 mU μl^{-1}) and 0.8 μl buffer (100 mmol l^{-1} $CaCl_2$ in 250 mmol l^{-1} Hepes, pH 6.0). To the hydrolysate were added 1.8 mol l^{-1} 1-ethyl-3-(3′-N,N′-dimethylaminopropyl)-carbodiimid (EDC) (15 μl in 50 mmol l^{-1} Hepes, pH 6.4), 27 mmol l^{-1} of the fluorescent dye Bodipy FL EDA™ (15 μl in 50 mmol l^{-1} Hepes, pH 6.4) and 15 μl Hepes (50 mmol l^{-1}, pH 6.4). The sample was then incubated in the dark for 21 h at 25°C. For reduction of Bodipy and salt content 55 μl of the derivatised sample were transferred into a 15-ml cap and diluted with 425 μl water. To the solution was slowly added 52.5 mmol l^{-1} sodium tetraphenylborate (425 μl in 1 mmol l^{-1} sodium phosphate buffer, pH 6.0). After mixing, 11 ml methylene chloride was added to the solution, which was mixed again and centrifuged for 4 min at 1912 ***g***. The aqueous phase was isolated and analyzed by capillary electrophoresis.

Analysis with capillary electrophoresis was carried out on a PACE™ MDQ system with a laser-induced-fluorescence detector (argon-ion laser with λ_{em}=488 nm) from Beckman Coulter (Munich, Germany). The fused-silica capillary used was purchased from CS-Chromatography-Service (Langerwehe, Germany) and had a total length of 50 cm (with the detection window at 40 cm) and an inner diameter of 50 μm. The separations were achieved with an electrolyte consisting of 90 mmol l^{-1} SDS in a solution of 90% v/v sodium phosphate buffer (20 mmol l^{-1}, pH 9.0) and 10% v/v methanol as organic modifier (5s-sample injection at 0.5 p.s.i. at 20°C and an applied voltage of 18 kV). The cathode was the outlet in all runs. For conditioning, the capillary was rinsed with 1 mol l^{-1} NaOH (15 min), 1 mol l^{-1} HCl (15 min), 1 mol l^{-1} NaOH (15 min), water (5 min) and electrolyte (10 min). Before each run it was rinsed with 200 mmol l^{-1} sodium dodecyl sulphate (1 min), 1 mol l^{-1} NaOH (1.5 min), water (1 min) and finally with electrolyte (2 min).

RESULTS AND DISCUSSION

In the following, 'developmental variation' (DV) is understood as that part of the phenotypic variation that cannot be explained by variations of the DNA sequence or variations of the macro-environment. Micro-environmental influences were reduced to a minimum by the experimental design but can never totally be excluded. Since such non-measurable environmental influences are believed to exert effects on the phenotype only on the long range *via* self-reinforcing circuitries, they are here included in DV. 'Development' covers the entire period from onset of cell division in the egg to death of the individual. The term 'epigenetic' is used in its broader sense, not just covering DNA and chromatin modifications.

Genetic identity of the marbled crayfish

Marbled crayfish reproduce by apomictic thelytoky, the development of female offspring from unfertilized eggs without meiosis, as revealed by cytological investigation of the oocytes from pre-vitellogenesis until cleavage (Vogt et al., 2004). The progeny of such apomictic parthenogens is generally regarded as being genetically uniform and identical to the mother, with the exception of random mutations. The clonal nature of the Marmorkrebs was recently demonstrated (Martin et al., 2007) using the highly sensitive microsatellite technique. By sequencing six micro-satellite loci, three of them being heterozygous, the authors revealed that batch-mates were genotypically identical with each other and the mother. They further revealed allelic identity in individuals from various generations of their laboratory population. In this study, the marbled crayfish were analysed using primers developed for *Procambarus clarkii* (see Belfiore and May, 2000), a species closely related to the Marmorkrebs (Scholtz et al., 2003). The size ranges and sequences of respective loci were different in the marbled crayfish and *Procambarus clarkii* [compare Belfiore and May (Belfiore and May, 2000) with Martin et al. (Martin et al., 2007)], excluding cross-contamination.

We have also tested the genetic uniformity of our experimental animals by investigation of nuclear microsatellite loci, using some of the primer combinations developed for *Procambarus clarkii*. Of the primer combinations tested, two (PclG-04 and PclG-26) yielded heterozygous alleles, which were investigated in depth and

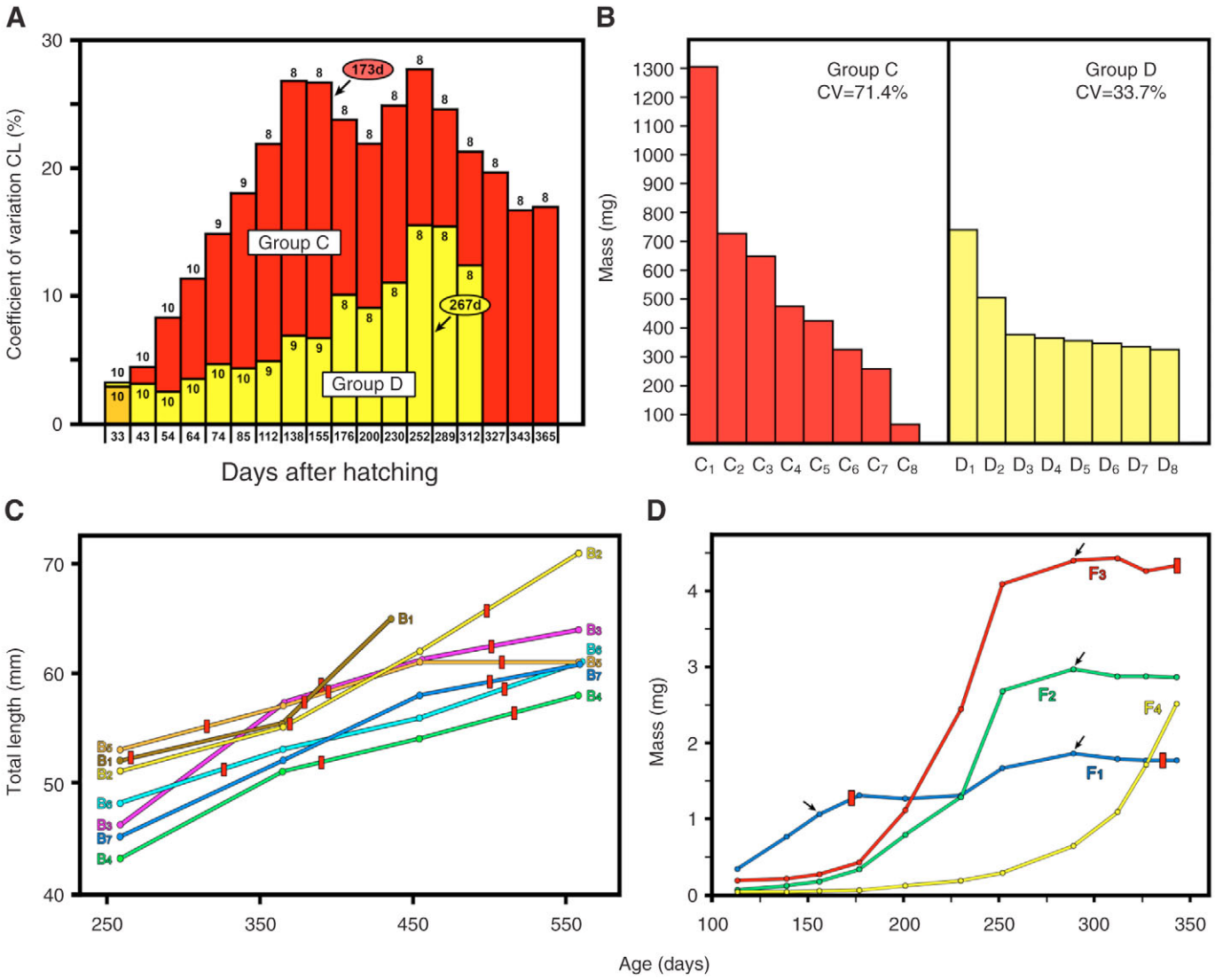


Fig. 2. Variation of growth and reproduction in adults. (A) Temporal development of variation of carapace length (CL), plotted as coefficient of variation, in batch-mates from dam $B_{3\text{-}1}$ that were divided into groups of ten (groups C and D) in the first day of stage 3 (before onset of feeding) and then kept under identical aquarium conditions. Figures on bars indicate number of specimens. Arrows indicate time (days) of first egg laying in group. (B) Variation of mass among batch-mates in groups C and D at day 164 of life. Note difference in ranges of variation and coefficients of variation (CV) between groups. (C) Variation in growth and reproduction of communally reared batch-mates (B_1–B_7) from dam B. Red vertical bars indicate time of oviposition. Note position changes of individuals in group with time. (D) Growth and reproduction of batch-mates (F_1–F_4) from dam $B_{3\text{-}1}$ reared together for 85 days and then kept individually under identical environmental conditions. Red vertical bars indicate time of oviposition. Arrows denote appearance of glair glands.

sequenced. All of the 21 marbled crayfish examined from our livestock and two further German populations showed the same allelic pattern, namely alleles of 156 bp and 172 bp at locus PclG-04 (repeat motif: TCTA) and alleles of 186 bp and 188 bp at locus PclG-26 (repeat motif: CA). The PCR products of our marbled crayfish were compared to the sequences of *Procambarus clarkii* (see Belfiore and May, 2000) (GenBank accession numbers AF290921 and AF290931, respectively), confirming that they differed in the sequences of the flanking regions as well as in the number of repeats. This way we can exclude possible cross-contamination. A batch of the sexually reproducing congeneric *Procambarus clarkii*, which served for comparison, included 11 homozygotes (229 bp/229 bp alleles) and 9 heterozygotes (193 bp/229 bp alleles) for 20 full siblings in the PclG-04 locus, which is in sharp contrast to our marbled crayfish.

Our microsatellite data support the results of Martin et al. (Martin et al., 2007) that batch-mates of the marbled crayfish are genetically identical. They further demonstrate genetic uniformity of our laboratory lineages and suggest genetic identity with the oldest German lineage from which the founder female of our laboratory population originated.

Variation of development and growth

The life cycle of the marbled crayfish can be subdivided into embryonic, juvenile, adolescent and adult periods (Vogt et al., 2004). At 20°C the embryonic period lasts ca. 20–25 days and is finished by hatching. In the following, the first eight postembryonic stages with prevailing spotted colour patterns are called juveniles and the later non-reproducing stages with increasingly marmorated patterns are referred to as adolescents. The adult period starts at the

earliest at stage 15 with the first egg laying. Postembryonic growth occurs in discrete steps by moulting and proceeds until death, usually comprising more than 20 stages. Growth of crayfish has a genetic component but is greatly influenced by the rearing temperature and the food (Jones et al., 2000; Reynolds, 2002). It is regulated by several growth promoting and inhibiting hormones, among them ecdysone, moult inhibiting hormone and methyl-farnesoate (Laufer et al., 2002; Vogt, 2002).

In our stock population, the speed of embryonic development was in some clutches homogeneous, in others heterogeneous, even under the same environmental conditions. For our experiments we used only specimens from homogeneously developing clutches, so the speed of development was always rather uniform during late embryonic development and also during the non-feeding juvenile stages 1 and 2, but became diverse after onset of feeding in stage 3, either on the maternal pleopods (Fig. 1A), in net-culture systems (Fig. 1B) or in micro-plates (Fig. 1E). For instance, in a natural mother–offspring association of dam A reared in a 30×20 cm aquarium, all juveniles passed stage 1 in 3–4 days and stage 2 in another 6–8 days. Stage 3, in contrast, was passed in 10 days by the fastest grower but only in 27 days by the slowest grower, although food was available in excess. Consequently, at day 35 after hatching 5 of 32 specimens were in stage 3 (Fig. 1C), 19 in stage 4, and eight already in stage 5 (Fig. 1D).

A similarly broad range of variation in development and growth was obtained when batch-mates were individually raised in the wells of a micro-plate (Fig. 1E), the simplest environment possible, or communally reared in net-culture systems. In the latter system we placed excess amounts of food at different sites of the vessels to guarantee that all juveniles had unlimited access to the food. Moreover, food uptake was regularly monitored by inspection of the stomach and gut through the rather transparent epidermis. We wish to emphasize that juvenile marbled crayfish are not filter feeders like the larvae of many decapod crustaceans (Anger, 2001). Instead, they take macroscopic food particles with their pereiopods from the ground and deliver them to the mouth like adult crayfish. Consequently, potential irregular distribution of microscopic debris in the water body is not expected to have an influence on the amount of food internalized.

The range of variation of growth usually increased in the period of adolescence among both communally (Fig. 1G and Fig. 2A,B) and individually (Fig. 2D) reared batch-mates. Again, by taking the measures listed above we could ensure that each individual had unlimited access to the food. Variation of growth was measured by determination of carapace length, total length and mass, but is best illustrated with respect to mass. For instance, in one of our experiments the heaviest individual of a communally reared group weighed approximately 20 times more than the lightest specimen at day 164 after hatching (Fig. 2B), although food was available in excess at any time. The broadest spectrum of variation of growth was obtained when adolescent batch-mates were reared without shelters, i.e. under conditions of social stress (Fig. 1F) (for details see Behaviour section below).

When batch-mates were divided into equal groups on the first day of stage 3 (i.e. before onset of feeding) and then raised in identical aquaria under the same environmental and nutritional conditions, the developing spectra of growth variation of the groups were usually not the same (Fig. 2A,B), indicating that group dynamics develop differently, despite the genetic identity of the group members and uniformity of the environment.

Increase of size and mass was also quite variable in the adult life period in both communally reared batch-mates (Fig. 2A,C) and siblings raised individually under the same conditions (Fig. 2D). In this period of life the situation is complicated by reproduction, as most females diminish food uptake during the breeding period, which starts with the development of externally visible glair glands. These glands produce the cement that is used to attach the eggs to the pleopods (Vogt and Tolley, 2004). Because of this close interdependence of growth and reproduction the range of variation can considerably fluctuate among adult batch-mates, either kept communally (Fig. 2C) or individually (Fig. 2D). Consequently, the relative group position of an individual can change considerably with time (Fig. 2C,D).

The grade of variation in development and growth differed considerably between rearing systems even when food and water conditions were kept constant. This became particularly apparent in an experiment with progeny of dam B, which were taken from the pleopods in stage 2 and then raised at constant water temperature of 21°C in three different rearing systems, a 60×30 cm aquarium equipped with shelter, a 30×20 cm aquarium equipped with a net only, and three 11×8 cm net culture vessels. At day 25 after hatching variation was highest in the aquarium with shelter: of the 51 sibs two were in stage 3, 24 in stage 4, 16 in stage 5 and nine in stage 6. In the smaller aquarium eight of 14 sibs were in stage 4 and six in stage 5, and in the net culture systems eight of 18 sibs were in stage 3 and ten in stage 4.

Our data suggest that development and growth can vary considerably among isogenic batch-mates even when reared in the same macro-environment with excess availability of food. These differences cannot be attributed to potential accumulation and defence of the food by one or more individuals of a group, as this behaviour was never observed. Moreover, such an argument would not explain the differences observed among individually raised batch-mates. Since variation in growth and development broadens markedly after onset of feeding, we assume that the individual decision, how much to feed and how often to feed and probably also slight differences in metabolism, which increase with time, are the main causes for this phenomenon. Our data also show that the range of variation can be modulated by differences in macro-environmental factors like space and shelter but also by group dynamics in the same environment.

Variation of life span and reproduction

A broad range of variation was also noted with respect to life-span and reproduction. Basic information on these traits is found elsewhere [for crayfish in general (Reynolds, 2002; Vogt, 2002) and for marbled crayfish (Vogt et al., 2004)]. The data given below refer to days after hatching.

The communally reared batch-mates B_1–B_8 had life-spans of 437, 626, 610, 571, 910, 568, 626 and 626 days, respectively. All specimens died during moulting, with the exception of B_2, B_7 and B_8, which were sacrificed for determination of DNA methylation. The range of the life-span of sibs died of natural causes varied between 437 and 910 days. The maximum age of a marbled crayfish so far recorded in our laboratory is 1154 days.

Female B_1 reproduced three times at days 157, 267 and 375, B_2 three times at days 160, 369 and 497, B_3 three times at days 168, 392 and 502, B_4 three times at days 183, 390 and 516, B_5 five times at days 315, 394, 507, 643 and 850, B_6 twice at days 328 and 507, B_7 once at day 500 and B_8 also once at day 531. Thus, the time from hatching to first spawning varied among these batch-mates between 157 and 531 days. Differences in reproduction become even more evident by comparing the reproductive success at a given day of life. For instance, at the age of 430 days female B_1

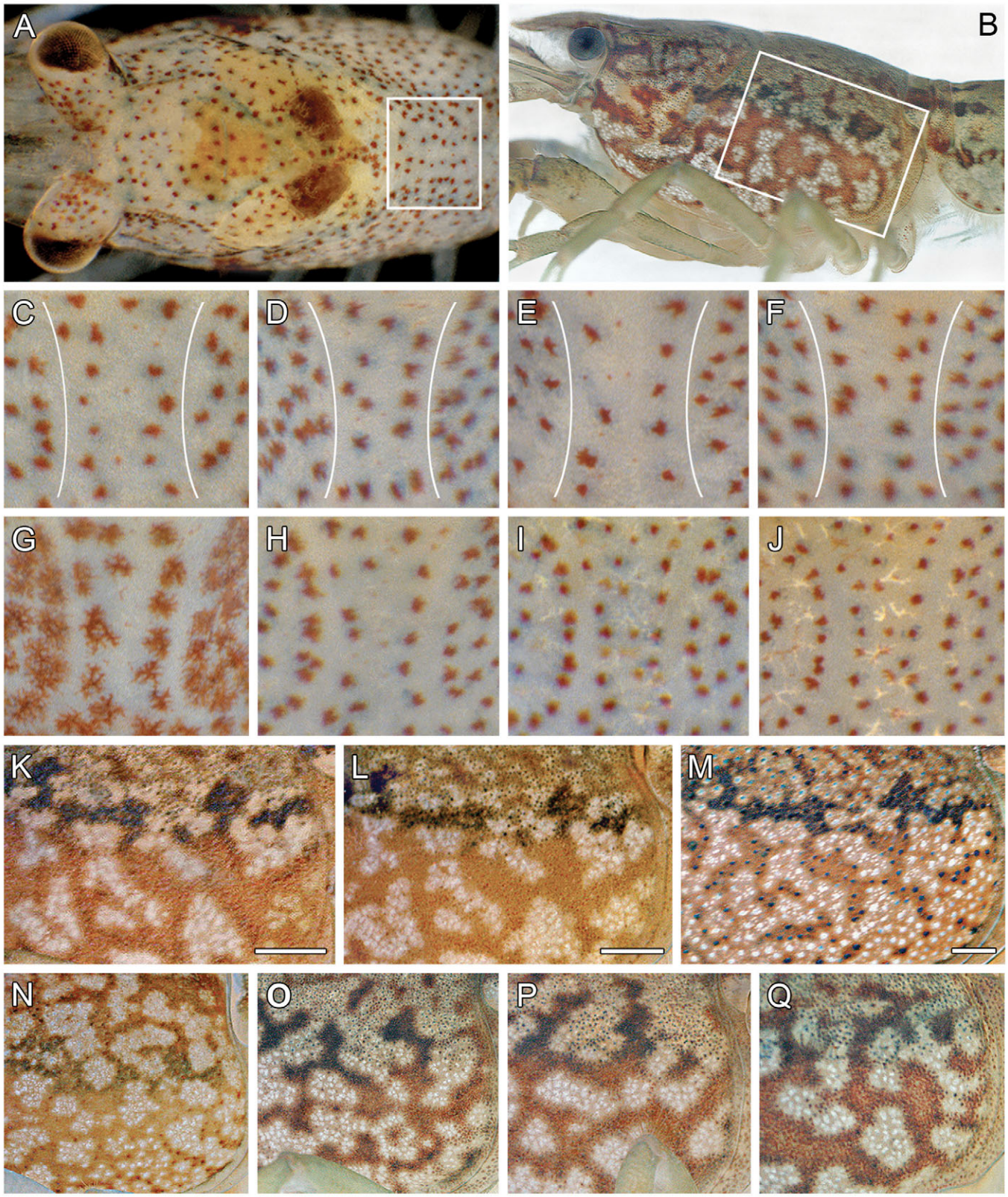


Fig. 3. Variation of coloration. (A,B) Body areas chosen in juveniles (A) and adults (B) for comparison of coloration. Frames indicate dorsal posterior (A) and posterio-lateral part of the carapace (B). (C–F) Areola (marked by white lines) of four stage-3 batch-mates, showing marked differences of pigmentation among individuals. (G–J) Pigmentation pattern of same specimen in juvenile stages 2 (G), 3 (H), 4 (I) and 5 (J), indicating that an existing pattern is not abruptly changed by moulting but gradually elaborated by addition of chromatophores. (K–M) Colour pattern of posterio-lateral carapace area of same adult at time of first reproduction (K), 124 days and 2 moults later (L), and 642 days and 6 moults later (M). The marmoration motifs are enlarged and modified with time but are still recognizable after more than 21 months. Scale bars, 2 mm. (N–Q) Colour patterns of posterio-lateral carapace area of dam B (N) and three mature daughters of the same batch (O–Q). Note striking differences in marmoration among all individuals.

had completed three reproductive cycles with a total of 219 stage-3 juveniles, the first independent life stage. Her sibs B_2, B_3, B_4 and B_5 had finished two reproductive cycles in the same period resulting in a total of 66, 153, 73 and 92 juveniles, respectively, whereas batch-mate B_6 had produced only one clutch of 52 juveniles. Sibs B_7 and B_8 had not yet reproduced at that time. The highest number of stage-3 juveniles so far produced in our laboratory by a single female per clutch is 421.

Our results demonstrate that life-span, onset of reproduction, frequency of reproduction and total reproductive success can vary considerably between batch-mates even when reared in the same aquarium and fed with a single food source in excess, suggesting that epigenetic phenomena can considerably influence fitness. Variation in reproductive traits was also observed among batch-mates of the parthenogenetic *Daphnia pulicaria* (Lajus and Alekseev, 2004). In these water fleas, some clone-mates produced resting eggs in a standardized environment but others did not.

Variation of coloration

The most extreme range of variation was recorded with respect to the eponymous coloration of the marbled crayfish (Fig. 3B), which is produced by two types of chromatophores and a diffuse background tanning. The red chromatophores appear at about 80% of embryonic development and contain red astaxanthin and, in later stages, also green and blue protein conjugates of astaxanthin. The white pigment cells appear first in stage-3 juveniles and include pteridines. The early juvenile stages display spotted motifs composed of individual pigment cells (Fig. 3A,C–J), which are transformed into marmorated motifs later on by multiplication of the chromatophores (Fig. 3B,K–Q). We would like to emphasize that crayfish cannot change their colour coat rapidly within seconds, minutes or hours by a 'physiological' colour change, which is typical of some shrimps or squids. Instead, they display a 'morphological' change of pigmentation, which involves quantitative changes of the chromatophores and qualitative changes of the pigments over weeks and months (Rao, 1985; Vogt, 2002). In early juvenile stages of the marbled crayfish the cuticle is transparent, and therefore, the pigment spots seen are the chromatophores of the epidermis. From approximately stage 7 the pigmentation pattern is more and more imprinted into the cuticle.

Investigation of the pigmentation pattern of the areola, a precisely lined area of the dorsal cephalothorax (Fig. 3A), in 20 offspring of dams A and B_1 from hatching to stage 7 revealed that each specimen has a unique pattern of chromatophores (Fig. 3C–F). This individual pattern is already well expressed in hatchlings and can be tracked through later life stages, although it becomes increasingly complex with time (Fig. 3G–J). Addition of both types of chromatophores to the existing pattern becomes particularly obvious from stage 5 on (compare Fig. 3H,J). The individuality of coloration is also maintained in adults, as shown by analysis of the posterio-lateral area of the carapace (Fig. 3B), the most intensely marmorated part of the adult body. Individual tracking of ten adults for a maximum of eight moulting cycles and 880 days revealed that during growth given motifs are enlarged in size whilst only moderately altered in structure by addition of chromatophores (Fig. 3K–M). Striking dissimilarities of the analysed area between dam B (Fig. 3N) and her mature offspring (Fig. 3O–Q) suggest that the marbling pattern is not inherited.

These results and the inspection of many more individuals indicate that marbled crayfish are individually identified throughout life, despite their clonal nature, principally permitting kin and group recognition. The extreme variation in marmoration is comparable only with the variability of leopard spots, zebra stripes, or human fingerprints and irises (Daugman and Downing, 2001; Murray, 2003), and may be best explained by Turing reaction–diffusion or Murray–Oster mechanochemical patterning mechanisms (Murray, 2003). This idea is supported by regularly observed differences in marmoration between right and left body sides (Fig. 6A), which is a typical outcome of those mathematical models. Uniformly colored marbled crayfish have not yet been found, suggesting that marmoration as such is genetically determined whilst the spatial distribution of the chromatophores is epigenetically regulated.

Variation of metric and meristic traits

Variations of carapace length and numbers of olfactory aesthetascs (Fig. 4A) and gustatory corrugated setae (CS) (Fig. 4B) were investigated in 182 juveniles raised communally in 11×8 cm net culture systems at 20°C. The CS are located along the inner margins of the propodus and dactylus of the chelae of pereiopods 1–3 (Fig. 1D) and include mechanoreceptors, broad-band amino acid receptors and further chemoreceptors (Altner et al., 1983; Vogt, 2002). The aesthetascs are located on the outer branches of the 1st antennae (Fig. 1D). Each aesthetasc includes more than 100 dendrites of olfactory receptor neurons (Sandeman and Sandeman, 2003; Vogt, 2002). Aesthetascs and CS appear first in juvenile stage 2. They are not only distinct morphological and functional units involved in perception of environmental information and probing of the food, but are also distinct evolutionary modules, as the aesthetascs are found in all crustaceans (Hallberg et al., 1992) whereas the CS are confined to the Decapoda (G.V., unpublished).

The ranges of the total numbers of aesthetascs per specimen varied between juvenile stages and batches (Fig. 4C). The same holds for the ranges of the total numbers of CS per specimen (Fig. 4D). Interestingly, for the same set of animals the patterns of variation differed considerably between these two traits, particularly with respect to their temporal development (compare Fig. 4C with D). In the case of the CS, variation was not only observed for their total number per specimen but also for their number on each of the pereiopods 1–3 (Fig. 4E). Specimens with regenerated or damaged antennae or pereiopods were excluded from scoring. Damage could be recognized by melanization, the typical wound healing of decapods (Vogt, 1999).

Differences in the numbers of aesthetascs among batch-mates are related both to different numbers of aesthetascs per article and to different numbers of aesthetasc-bearing articles per antenna. The distal-most article always carries three unpaired aesthetascs, which is not varied. The following articles carry usually one pair of aesthetascs in the juvenile stages 2–4 and a maximum of two pairs in the following stages, including the adults (Fig. 4A) (Vogt and Tolley, 2004). In these articles the number of aesthetascs can be varied from one to four. Variation of the number of CS is mainly related to differences in length of the rows of these sensory structures on the propodus and dactylus of each of the pereiopods 1–3 (Fig. 4B). Interestingly, differences in the numbers of aesthetascs between individuals are expected to have considerable consequences for brain structure, because each of the many chemoreceptors of an aesthetasc is individually integrated into the olfactory lobe of the deutocerebrum, resulting in enlargement of this brain area (Mellon and Tewari, 2000; Sandeman and Sandeman, 2003). Such brain differences may finally lead to differences in individual performance.

In order to analyse the dependence of the traits CS, aesthetascs (Ae) and carapace length (CL) from juvenile stage and batch in more

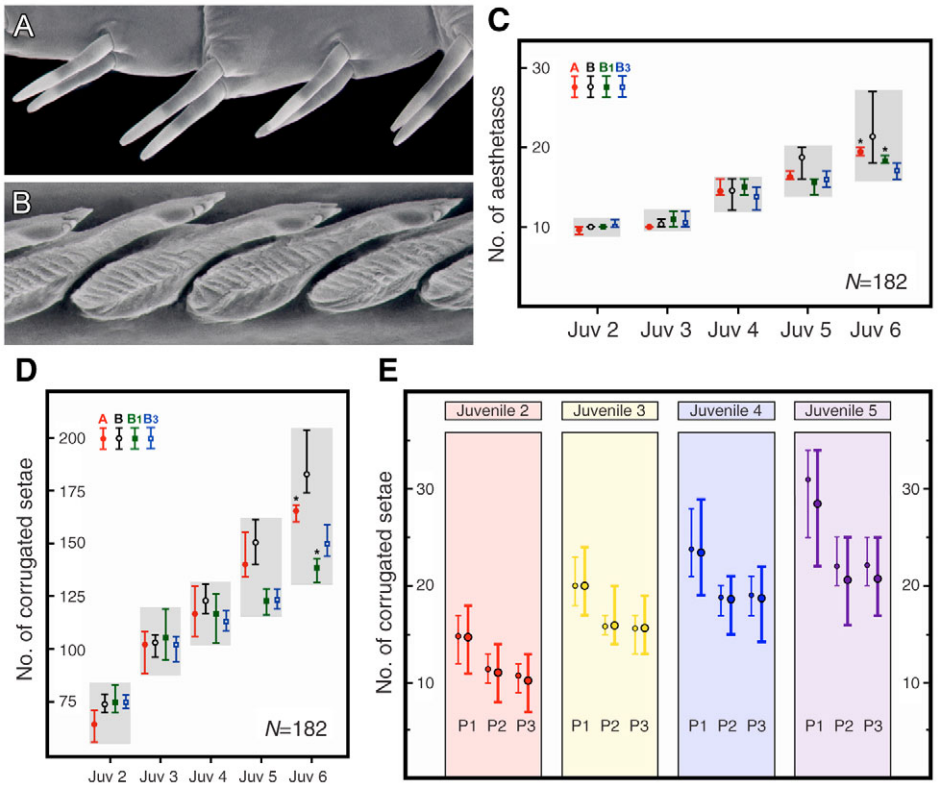


Fig. 4. Variation of sense organs. (A) Scanning electron micrograph of olfactory aesthetascs in an adolescent. (B) Scanning electron micrograph of gustatory corrugated setae in an adolescent. (C) Means and ranges of the numbers of aesthetascs per specimen, plotted per stage (Juv 2 to Juv 6) and batch (from dams A and B and daughters B_1 and B_3). Juveniles were removed from the maternal pleopods in stage 2 and then reared under identical conditions in net culture systems. Shaded areas indicate ranges of all batches. *Only three individuals analysed in these groups. (D) Means and ranges of the numbers of corrugated setae per specimen (same specimens as in C), plotted per juvenile stage and batch. Note differences in variation among aesthetascs in C and corrugated setae in D. (E) Means and ranges of the numbers of corrugated setae on pereiopods 1, 2 and 3 (P1–P3), plotted per juvenile stage. Small circles and thin bars represent offspring of a single female (dam B) and large circles and thick bars pooled data of all batches (from dams A, B, B_1 and B_3).

detail, a bifactorial ANOVA with interaction was applied. The idea behind the model is that the influence of stage and of batch could combine multiplicatively if size and therefore the numbers of sensory organs were determined by an early factor such as yolk supply. The results are presented in Table 1 (these log-based values are very similar to corresponding results obtained without using logarithms). The data indicate that for each of the three variables both main effects and interaction term are highly significant. This means that there is a difference among the juvenile stages, which is trivial, that there is a significant multiplicative difference in the means between the different batches, which may be caused by differences in yolk supply, feeding behaviour or group dynamics among batches, and that these two effects do not fully explain the observed dataset, as the interaction term is also significant. Different batches show a varying deviation from the mean in the different juvenile stages. These differences cannot be explained by a single additive or multiplicative effect, but suggest an unpredictable and/or nonlinear mechanism for generation of variability.

The variances of the 20 sub-samples (different stages and batches) were compared by a Bartlett test for log(CL) and log(CS) and were shown to be highly significant [P=0.002247 for log(CL) and $P=7.415\times10^{-5}$ for log(CS)]. Fig. 5 shows the estimated geometric standard deviations with their corresponding 95%-confidence limits. The confidence limits overlap in most cases so that no individual differences can be established. However for CL and CS the relative variation seems to be larger in all stages for batch B_1. We therefore conclude that there is a maternal influence on the development of the genetically identical progeny but that this influence is not deterministic but rather expressed in the variation of the offspring. On the other hand the relative errors show no obvious change throughout the different juvenile stages. Based on all data the geometric standard deviation was estimated by transforming MSE of the corresponding ANOVA models to be s_g(CS)=1.049, s_g(Ae)=1.060 and s_g(CL)=1.041, and thus the within-batch variation is about 5% for all three parameters.

In a more detailed analysis of the dependence structure we have investigated the dependence of the three variables CS, carapace length and aesthetascs, given batch and stage. The data in Table 2 show that both the dependence of CS and carapace length and

Table 1. ANOVA for the traits aesthetascs (Ae), corrugated setae (CS) and carapace length (CL)

	d.f.	SS	MSE	F	P
log(Ae)					
Stage	4	11.9754	2.9939	890.509	$<2.2\times10^{-16}$***
Batch	3	0.2323	0.0774	23.033	1.802×10^{-12}***
Stage:batch	12	0.4248	0.0354	10.529	3.183×10^{-15}***
Residuals	162	0.5446	0.0034		
log(CS)					
Stage	4	12.7192	3.1798	1403.999	$<2.2\times10^{-16}$***
Batch	3	0.2871	0.0957	42.262	$<2.2\times10^{-16}$***
Stage:batch	12	0.5206	0.0434	19.154	$<2.2\times10^{-16}$***
Residuals	162	0.3669	0.0023		
log(CL)					
Stage	4	6.9801	1.7450	1077.368	$<2.2\times10^{-16}$***
Batch	3	0.4987	0.1662	102.625	$<2.2\times10^{-16}$***
Stage:batch	12	0.2392	0.0199	12.306	$<2.2\times10^{-16}$***
Residuals	162	0.2624	0.0016		

d.f., degrees of freedom; SS, sums of squares; MSE, mean square error; F, variance ratio; P, probability; ***highly significant (P<0.001).

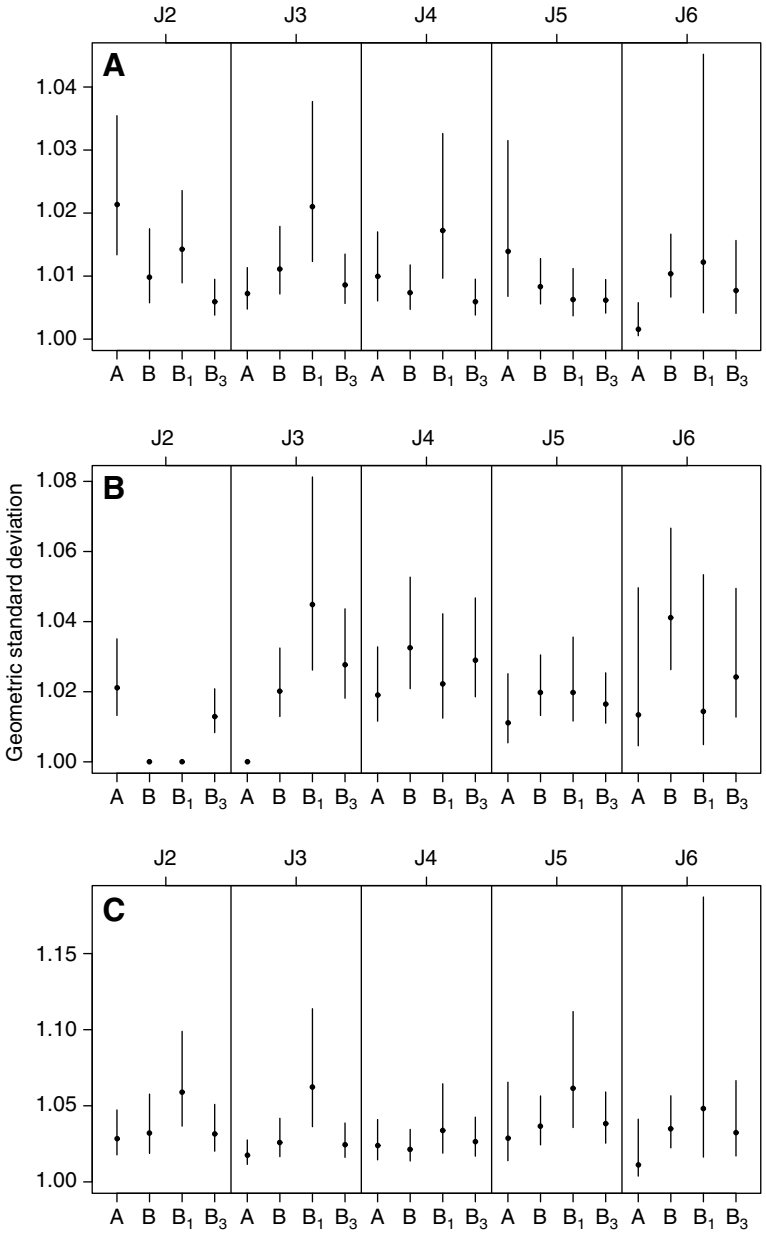


Fig. 5. Geometric standard deviations s_g=exp{sd[log(x)]} of corrugated setae (A), aesthetascs (B) and carapace length (C) with 95% confidence limits. Data are split according to batches (A, B, B_1, B_3) and juvenile stages (J2, J3, J4, J5, J6). The normality approximation in s.d. confidence limits does not hold for those aesthetasc data sets (in B) that showed no variation, and therefore no confidence limits are given in these cases. The dots correspond to the estimated standard deviation. s_g is a measure of relative variation and is approximately 1+CV. Note the typically higher variation in batch B_1.

the dependence of CS and aesthetascs are highly significant, indicating that the driving forces of the random variation do not only affect individual traits, but also the whole animal. On the other hand, there is no significant dependence between carapace length and aesthetascs, and the reduction in residual mean squared error with respect to the previous ANOVA models is very small, suggesting that the sources of randomness cannot just be linked to a single cause but to more complex mechanisms.

We suppose that the variation in the numbers of aesthetascs and corrugated setae among batch mates are caused by slight differences in the expression and reaction–diffusion patterns of morphogenetic factors. This idea is inferred from results by Dohle et al. (Dohle et al., 2004), who showed that during limb development morphogens can communicate information to members of different cell lineages. In other words, gene expression is independent of stereotyped differential cleavage in early development of crayfish. Such differences in reaction–diffusion-like morphogenetic mechanisms would also explain the semi-independent behaviour of the traits investigated.

Variation of behaviour

Communally and also individually raised batch-mates displayed a broad range of variation with regard to particular behavioural elements. Basic data on the behaviour of crayfish are published elsewhere (Gherardi, 2002; Lundberg, 2004). For instance, as soon as the juveniles could move, which was in stage 2, they developed differences in locomotion. They even developed differences in social behaviour since some specimens preferred to be solitary in net culture while others tended to aggregate. Many other examples were observed in adolescents and adults. For instance, some batch-mates moulted in the morning but others in the evening or during the night. Most specimens rested under the shelters in a sitting position but some preferred always to lie on the back. Oviposition usually occurred during the night but some individuals preferred to lay the eggs during the day. Some dams fed during the periods of breeding and brooding of the juveniles but others did not touch the food at all. Most of the berried females removed decaying eggs from the clutch but some did not. These behavioural differences were quite obvious but we also assume variation in other less easily visible aspects, such as quantity of food uptake as discussed above. Since most of these behavioural variations are related to differences in individual decisions rather than to social interactions and since they are also found among individually raised batch-mates they are treated as a part of DV rather than of the macro-environment.

The most dramatic establishment of differences in behaviour among batch-mates was observed when groups of five freshly moulted and size-matched stage-6 sibs with neutral agonistic behaviour were placed in 30×20 cm aquaria without shelter (replicated 3 times). In the next 34 days a social hierarchy was gradually established, beginning at stage 7, the first stage with sclerotized claws suitable for agonistic interactions. This social hierarchy finally included one dominant, one subdominant and three subordinates or one dominant, two subdominants and two subordinates. Establishment of dominance was paralleled by the development of increasingly offensive and aggressive behaviours (Lundberg, 2004) of the dominant and increasingly defensive and avoiding behaviours of the subordinates. Such pronounced hierarchies were not established when shelters were available, corroborating the importance of shelters for the culture of adolescent and adult crayfish (Gherardi, 2002).

Interestingly, the dominants grew much faster than the subordinates (Fig. 1F), although all specimens had unlimited access to the food and fed regularly, as revealed by inspection of the stomach and intestine through the transparent epidermis. These observations suggest that even in size-matched clone-mates, slight initial

Table 2. ANOVA testing for conditional dependence of the traits aesthetascs (Ae), corrugated setae (CS) and carapace length (CL)

	d.f.	SS	MSE	*F*	*P*
log(Ae) *versus* CL					
Stage + batch	19	12.6325	0.6649	196.8912	$<2.2\times10^{-16}$***
CL	1	0.0010	0.0010	0.2868	0.593
Residuals	161	0.5437	0.0034		
log(CS) *versus* CL					
Stage + batch	19	13.5269	0.7119	352.891	$<2.2\times10^{-16}$***
CL	1	0.0421	0.0421	20.863	9.764×10^{-6}***
Residuals	161	0.3248	0.0020		
log(Ae) *versus* CS					
Stage + batch	19	12.6325	0.6649	219.206	$<2.2\times10^{-16}$***
CS	1	0.0563	0.0563	18.567	2.846×10^{-5}***
Residuals	161	0.4883	0.0030		

d.f., degrees of freedom; SS, sums of squares; MSE, mean square error; *F*, variance ratio; *P*, probability; ***highly significant ($P<0.001$).

physiological or behavioural differences can finally result in a broad range of phenotypes, probably by self-reinforcing circuits involving behaviour, sense organs, brain, hormone system and metabolism. This idea is corroborated by Song et al. (Song et al., 2007), who observed higher survival of brain cell precursors in dominant crayfish compared to subordinates. The relationship of dominance and faster growth is well known from bisexual crayfish species but is usually attributed to genetic differences in food conversion and aggression (Reynolds, 1989). Behavioural variations among genetically identical organisms kept in a constant environment were also observed for cloned pigs (Archer et al., 2003b).

Variation of characters among body sides

Right–left differences are traditional organismic indicators of the epigenetic proportion of a trait, as the genes are the same in both body sides. The most frequently measured parameter is fluctuating asymmetry (FA), the numerical difference of a trait between right and left side (Debat and David, 2001; Dongen, 2006; Lajus et al., 2003; Møller, 2006; Nijhout and Davidowitz, 2003). However, FA has to be regarded as a special aspect of DV, because right and left body structures are linked to each other by the nervous system and circulation, which may allow correction of asymmetries. In the marbled crayfish right–left differences were observed for all traits investigated, being most prominent for coloration. In each specimen examined the marmoration patterns of both body sides were far away form being mirror-symmetric (Fig. 6A), emphasizing that body pigmentation has a high epigenetic component.

FA of the aesthetascs and corrugated setae varied considerably between juvenile stages and also among batches (Fig. 6B). It fluctuated roughly between +3% and –3%, in extreme cases between +6 and –6%. In the entire population of the 168 specimens investigated FA was close to zero for both sense organs (aesthetascs: –0.34%; corrugated setae: –0.32%), indicating the good state of health of our laboratory population (Møller, 2006). Interestingly, FAs of both traits were not correlated (Fig. 6B). Common to both traits, however, was shifting of FA between body sides throughout development, suggesting that asymmetries are indeed corrected when they exceed a certain range. This idea is corroborated by longitudinal analyses of individual FAs, as exemplified in Fig. 6C. In that specimen, FA of the aesthetascs changed body sides twice between stages 6 and 14, as did FA of the CS of pereiopod 2.

Our data also demonstrate that the traits investigated behaved as semi-independent modules. For example, in the individual analyzed in Fig. 6C, growth increments of the carapace were almost identical (1.5 mm *versus* 1.4 mm) in stages 9 and 10, whereas the increases in the numbers of aesthetascs (5 *versus* 11) and CS (16 *versus* 60) were quite different in the same periods. The modular architecture of animals has received great attention in the last years, because developmental modules may be uncoupled under certain circumstances and act as quasi-independent units of evolutionary transformation, possibly leading to new phenotypes or even new body plans (Franz-Odendaal and Hall, 2006; Schlosser and Wagner, 2004).

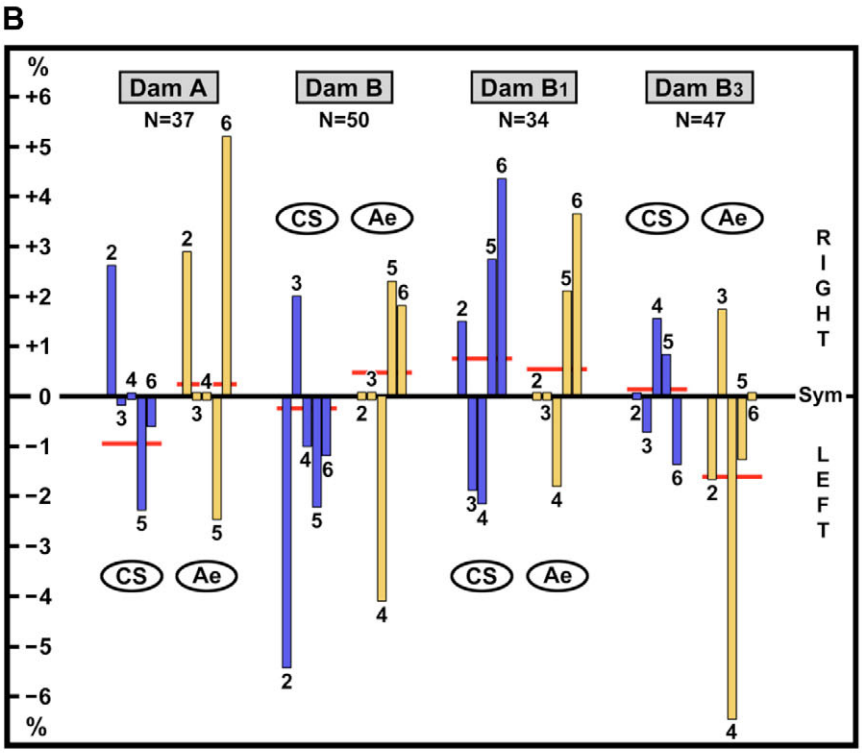
Variation of global DNA methylation

Recent studies on cloned and inbred mice and monozygotic human twins revealed that the epigenotype, which includes DNA methylation, histone modifications and modifications of regulatory proteins on the DNA, can vary between genetically identical individuals and may be associated with phenotypic differences (Fraga et al., 2005; Ohgane et al., 2001; Whitelaw and Whitelaw, 2006). In order to test if differently grown batch-mates of the marbled crayfish also show differences in the epigenetic code, we measured global methylation of cytosines in the DNA of the hepatopancreas and the abdominal musculature, using an improved and highly sensitive analytical technique recently developed by us (Schiewek et al., 2007). This method allows the determination of very low genome-wide methylation levels (<1%) from DNA samples as small as 1 μg. The methylation level is calculated with the equation [5mdC]/([5mdC]+[dC]).

The data obtained from two communally raised batches revealed that global DNA methylation varied among batch-mates and also among tissues (Fig. 6D). In four 188-day-old adolescents with total lengths of 3.2 cm to 4.2 cm (mean: 3.58 cm) and mass of 0.68 g to 1.71 g (mean: 1.01 g), methylation varied between 1.75% and 1.94% (mean 1.86%) in the hepatopancreas and between 1.96% and 2.09% (mean 2.01%) in the abdominal musculature. In three 626-day-old adults of 6.2 cm to 7.0 cm total length (mean: 6.7 cm) and mass of 6.04 g to 10.32 g (mean: 7.99 g) methylation varied between 1.52% and 1.78% (mean: 1.65%) in the hepatopancreas and between 1.77% and 1.92% (mean: 1.84%) in the musculature.

Our data demonstrate within-batch variation of global DNA methylation in the hepatopancreas and abdominal musculature but do not show a correlation with growth, although there seems to be a slight tendency that the fastest growers have the lowest methylation values in the abdominal musculature. At present, it is not even known whether methylation plays a similar role in regulation of the genes in crayfish as in vertebrates. Our data clearly indicate, however, that the DNA of crayfish is methylated not only during juvenile development but also in reproducing adults, which is in contrast to *Drosophila*, where the DNA is methylated in the embryos only. But even in this fly, which is also an exceptional case with respect to other aspects such as the size of the genome, DNA methylation was shown to play a key role in differentiation (Mandrioli and Borsatti, 2006).

We would also like to emphasize that small differences in global DNA methylation can have great consequences for the phenotype. For instance, in *in vitro* fertilized bovine fetuses fetal overgrowth



C

Stage	CL	Aesthetascs		Corrugated setae					
		A1-r	A1-l	P1-r	P1-l	P2-r	P2-l	P3-r	P3-l
Juv 6	?	9	10	35	33	?	23	?	25
Juv 7	4.9	12	12	47	42	27	28	30	29
Juv 8	5.3	15	14	?	?	?	?	?	?
Juv 9	6.1	17	16	54	53	39	38	37	36
Juv 10	7.6	18	20	58	55	41	40	40	39
Juv 11	9.0	24	25	72	67	50	48	48	48
Juv 12	10.1	?	?	76	73	?	?	?	?
Juv 13	11.9	37	41	91	86	?	70	69	71
Juv 14	12.5	39	41	92	91	70	71	71	72

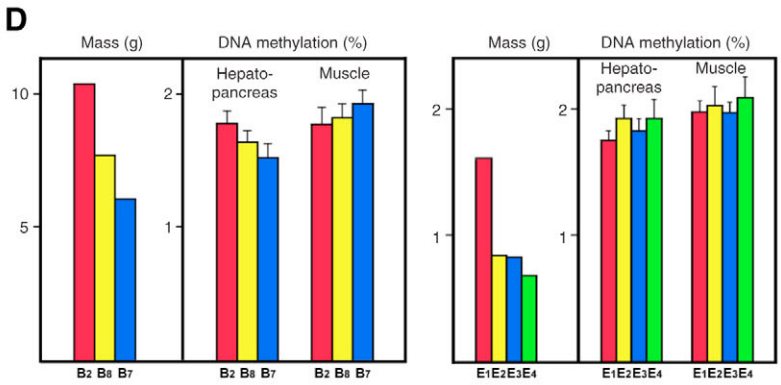


Fig. 6. Variation of epigenetic markers. (A) Right–left differences of marmoration pattern on cephalothorax of mature female. (B) Variation of fluctuating asymmetry (FA) of corrugated setae (CS) and aesthetascs (Ae) in 168 stage-2 to stage-6 juveniles (stages indicated by numbers on bars) from dams A, B, B_1 and B_3. FA is expressed as % deviation from perfect symmetry (Sym), either to the right (+) or to the left (–). Red horizontal bars indicate mean FAs of batches. *N*, number of individuals investigated per batch. Note independence of fluctuation of both traits during ontogeny and variation among batches. (C) Alteration of carapace length (CL), number of aesthetascs and number of corrugated setae in a single crayfish through nine life stages. Development of the three traits is not strictly correlated and FA of the sense organs can fluctuate between body sides. A1-r and A1-l, right and left 1st antennae; P1-r to P3-l, right and left pereiopods 1, 2 and 3. (D) Variation of body mass and global DNA methylation of the hepatopancreas and abdominal musculature in three communally reared adult batch-mates (B_2, B_7, B_8) at the age of 626 days (left) and four adolescents (E_1–E_4) at the age of 188 days (right). Each DNA sample was measured at least 20 times. Bars indicate +s.d. Variation of mass and global DNA methylation is apparently not correlated.

and associated endocrine changes were related to only 11.2% deviation from normal methylation values of the liver (Hiendleder et al., 2006). In the hepatopancreas of our crayfish, which is functionally comparable to the liver of vertebrates (Vogt, 2002), the difference of methylation between the heaviest and lightest specimen was 17.1% in the adult group and 11.3% in the adolescents. This first study on global DNA methylation in the marbled crayfish and its variation should be followed by more specific research on the methylation of genes that regulate growth in crayfish (see Development and growth section) and on potential correlations with growth differences.

CONCLUSIONS

The following conclusions can be drawn from our results: (1) Identically raised clone-mates of the marbled crayfish differ from each other as a consequence of DV, demonstrating that even in the same environment, genotype-to-phenotype mapping is not one-to-one but one-to-many. This phenomenon, which illustrates the power of epigenetics, may also explain the phenotypic differences observed among cloned animals, plants and monozygotic human twins. (2) DV can change randomly during a lifetime, introducing components of chance into individual life history and population dynamics. These observations make the presumed relevance of

'noise' in aging and susceptibility to diseases in animals and man plausible. (3) DV is apparently not created by a single embryonic event but can be produced in all life stages by a variety of stochasticity generators. These may include differences in provision of the eggs with nutrients (production of heterogeneous clutches), reaction-diffusion-like patterning mechanisms (variation in coloration and number of sense organs) or nonlinear, self-reinforcing circuitries involving behaviour and metabolism (variation in growth, reproduction and life-span). (4) Olfactory and gustatory sense organs behaved as semi-independent modules with respect to FA, the deviation from perfect symmetry. Moreover, FA was repeatedly corrected during development. (5) Global DNA methylation, a molecular overall marker of the epigenetic state of an individual, also varied among batch-mates but this variation could not be correlated with variation of the life history parameters or the morphological traits.

PERSPECTIVES

DV is apparently a basic biological principle, because it has been documented in systematically distant animal taxa, plants and man. This phenomenon may be particularly pronounced in the marbled crayfish and easier to analyze than in other species, making this crayfish a suitable laboratory model for research on the role of epigenetics in shaping of the phenotype and on the analysis of self-reinforcing circuits including behaviour and metabolism. Since the marbled crayfish can tolerate a very broad range of experimental conditions it is also recommended for research on environmental epigenomics. Investigation of the various generators of DV and their modifiability by environmental factors are prime goals of our future research.

Although we have no evidence so far that any of the recorded variations are adaptive or heritable, we would like to end with some speculations in order to stimulate further research on the role of DV in evolutionary biology. DV may be of particular significance to clonal organisms and invasive pests, because it increases their chance to persist in fluctuating or new environments by *a-priori* provision of a broader spectrum of phenotypes. In other words, DV may keep clonal species in the game when the environment changes. Since the phenotype is the principal target of natural selection, DV may even act as a general evolution factor by contributing to the production of a broader range of phenotypes that may occupy different ecological micro-niches, paving the way to speciation *via* differential mutagenesis or epigenetic inheritance (Loxdale and Lushai, 2003; Rakyan and Beck, 2006; Schlichting and Pigliucci, 1998; Whitelaw and Whitelaw, 2006; West-Eberhard, 2005; Zakharov, 1993).

Meanwhile, some further examples of developmental variation (DV) were found among identically raised batch-mates of the marbled crayfish. These variations concern the symmetry of internal organs, movement patterns and social behaviours, and the response to environmental toxicants. For instance, the sternal artery, which connects the heart to the ventral thoraco-abdominal artery, can be either paired and bilateral symmetric, or unpaired and right or left asymmetric, as revealed by serial sectioning of more than 100 juveniles (Vogt et al., manuscript submitted for publication). Movement patterns and social behaviours were shown to vary considerably among stage-2 batch-mates raised from *in vitro* cultured eggs (Vogt, 2008). And the duration of embryonic development, hatching success, growth of the juveniles, and grade of malformation of the appendages varied considerably among sibs exposed in 12-well micro-plates to 100 $\mu g\,l^{-1}$ 17α-methyl testosterone (Vogt, 2007).

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