High degree of non-genetic phenotypic variation in the vascular system of crayfish: a discussion of possible causes and implications

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Abstract

In this study, the hemolymph vascular system (HVS) in two cambarid crayfishes, i.e. the Marbled Crayfish, *Procambarus virginalis* Lyko, 2017 and the Spiny Cheek Crayfish, *Faxonius limosus* (Rafinesque, 1817), is investigated in regard of areas of non-genetic phenotypic variation. Despite their genetic identity, specimens of *P. virginalis* show variability in certain features of the HVS. Thus, we describe varying branching patterns, sporadic anastomoses, and different symmetry states in the vascular system of the marbled crayfish. We visualize our findings by application of classical and modern morphological methods, e.g. injection of casting resin, micro-computed tomography and scanning electron microscopy. By comparing our findings for *P. virginalis* to the vasculature in sexually reproducing crayfishes, i.e. *F. limosus* and *Astacus astacus*, we discuss phenotypic variation of the HVS in arthropods in general. We conclude that constant features of the HVS are hereditary, whereas varying states identified by study of the clonal *P. virginalis* must be caused by non-genetic factors and, that congruent variations in sexually reproducing *F. limosus* and *A. astacus* are likely also non-genetic phenotypic variations. Both common causal factors for non-genetic phenotypic variation, i.e., phenotypic plasticity and stochastic developmental variation are discussed along our findings regarding the vascular systems. Further aspects, such as the significance of non-genetic phenotypic variation for phylogenetic interpretations are discussed.

Keywords Evolutionary morphology · Phenotypic plasticity · Circulatory system · Heart · Artery

Introduction

Intraspecific variability of arthropod circulatory systems

The circulatory systems of arthropods have been shown to be subject to a high degree of intraspecific variability. Such intraspecific variability can be found all across the arthropod tree of life and within all constituents of the hemolymph circulatory system. In Xiphosura, various prominent regions of both, the vascular system and the lacunar system have been shown to vary intraspecifically, as well as even the number of ostia (Göpel and Wirkner 2015). In arachnids, a significant

degree of intraspecific variability was found especially in the supply of the prosomal ganglion and adjacent structures (Huckstorf et al. 2015; Runge and Wirkner 2016). The same can be reported for the vascular supply in at least scutigero-morph chilopods (unpublished data). Within crustaceans, the Decapoda so far have been studied most extensively in regard to intraspecific variability of vascular morphology (Baumann 1921; Imafuku 1993; Vogt et al. 2009; Keiler et al. 2013, 2015, 2016). Most prominently, the descending artery, one of the major cardiac arteries and responsible for the supply of the entire ventral cephalothorax, can be either paired or unpaired passing the gut on either its right or left side (Imafuku 1993; Vogt et al. 2009). The relative frequency of the different morphological conditions (i.e. right-sided, left-sided, or symmetrical descending artery), appears to differ greatly between decapod taxa and in taxa with asymmetric habitus, often is independent from handedness (Imafuku 1993). While the question of whether such intraspecific variability is genetically determined could not be answered in other species, the parthenogenetic marbled crayfish showed the same variability in its genetically identical offspring (Vogt et al. 2009; Gutekunst

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et al. 2018). These findings point towards non-genetic phenotypic variation as cause for this kind of variability (Vogt et al. 2009).

Phenotypic plasticity and stochastic developmental variation

Phenotypic plasticity generally refers to different phenotypes (i.e. different morphological, physiological and/or behavioral traits) of the same genotype in response to changing environmental conditions to which the organism is exposed (West-Eberhard 1989; Kelly et al. 2012; Pelster and Burggren 2018). Phenotypic plasticity can be induced for instance by abiotic environmental factors (e.g. temperature) or by presence of other organisms such as predators, e.g. in water fleas (mediated through chemical cues; Tollrian 1993, 1995). As direct response to environmental factors, phenotypic plasticity is more frequent and thus considered advantageous in changing environments (Langerhans and DeWitt 2002; Ghalambor et al. 2007; Kelly et al. 2012; Chevin and Hoffmann 2017; Pelster and Burggren 2018).

Another aspect of non-genetic phenotypic variation is stochastic developmental variation (Vogt 2015, 2017, 2020; Angers et al. 2020). Although subject to the same environmental conditions, genetically identical organisms can develop different phenotypes due to stochastic alterations of the epigenome (Leung et al. 2016; Atlasi and Stunnenberg 2017; Angers et al. 2020; Vogt 2020). Thus, stochastic developmental variation, unlike phenotypic plasticity, is no direct response to specific change of environmental factors but considered a bet-hedging strategy (Simons 2014; Furness et al. 2015; Vogt 2015; Leung et al. 2016; Angers et al. 2020).

Environmentally induced phenotypic plasticity and stochastic developmental variation, however, are no mutually exclusive strategies but complementary sources of phenotypic variation (Vogt 2020). While phenotypic plasticity is considered advantageous in habitats with rather predictable environmental changes, stochastic developmental variation is considered a diversifying bet-hedging strategy suited for less predictable or unpredictable environments (Leung et al. 2016). In natural environments, which cannot be characterized binarily as either predictable or unpredictable, the coexistence of phenotypic plasticity and stochastic developmental variation is likely to be a beneficial compromise for environments with both predictable and unpredictable changes of conditions (Leung et al. 2016).

The marbled crayfish as ‘model organism’ for phenotypic variation

The parthenogenetic reproduction makes *Procambarus virginalis* unique among the decapod crustaceans (Martin et al.

2007; Vogt et al. 2008) and interesting as object of research. The marbled crayfish *P. virginalis* first appeared in German aquarium trade in the mid-1990s (Martin et al. 2010). Due to its exclusively parthenogenetic reproduction with only females present it soon gained the interest of researchers (Scholtz et al. 2003; Martin et al. 2007). After it had been shown that *P. virginalis* is a triploid form of the North American crayfish *Procambarus fallax* (Hagen, 1870) (Martin et al. 2016; Gutekunst et al. 2018), the marbled crayfish has recently been described as separate species by Lyko (2017). The mode of parthenogenesis in *P. virginalis* is exclusively apomictic. In this kind of parthenogenesis, in which meiosis is suppressed and diploid (or in this case triploid) eggs are produced by mitosis only (Simon et al. 2003), all offspring is both genetically identical among themselves and to the mother (Martin et al. 2007; Gutekunst et al. 2018). Furthermore, *P. virginalis* has a relatively short generation time (compared to other decapods), features high robustness regarding rearing conditions and thus is well suited for the study of both phenotypic plasticity and stochastic developmental variation (Vogt 2008, 2020; Vogt et al. 2008).

In the case of *P. virginalis*, Vogt et al. (2008) observed phenotypical variation of life history traits, fitness, pigmentation as well as dispersion of setae and antennal aesthetascs.

Previously, we recorded the varying symmetry states of descending artery as an example of stochastic developmental variation (Vogt et al. 2009). More recently, we have described the gross morphology of the HVS of *P. virginalis* based on its constant patterns (Scholz et al. 2018), whereas in the present work, we investigate the hemolymph vascular system of the marbled crayfish in regard to varying patterns of the vascular system as the result of non-genetic variation.

We describe different kinds of intraspecific variability of vascular morphology exemplified by visualizations of alternative vascular morphologies in several individuals of *P. virginalis*. By comparing these data with that of the two sexually reproducing species *Faxonius limosus* (Rafinesque, 1817) (own investigation) and *Astacus astacus* (Linnaeus, 1758) (data from the literature; Baumann 1921), putative variable patterns in vascular morphology in crayfish in general are identified. As a starting point for the investigation of stochastic developmental variation (as well as phenotypic plasticity) of internal morphology in decapod circulatory systems, this phenomenon is discussed in the framework of evolutionary morphology.

Materials and methods

Species studied

Procambarus virginalis Lyko, 2017 and *Faxonius limosus* (Rafinesque, 1817)

Adult specimens of *P. virginalis* were bought at a pet shop, and reared individually in 6-L aquaria filled with tap water at room temperature (22 ± 3 °C); 48 specimens were studied. For histology, one juvenile (3 mm carapace length) as well as twelve recently hatched animals were dissected. For injection preparations, specimens with a carapace length of at least 1 cm were used. Adult specimens of *F. limosus* with a carapace length of about 4.5 cm were caught from a lake near Rostock (Hohensprenzer See) in November 2009. These animals were kept together in a 300-L aquarium filled with tap water at 5 °C and equipped with shelters; 22 specimens were studied. All animals at any life stage were fed ad libitum with “Novo Crabs” food chips (JBL, Neuhofen, Germany).

Histology

The specimens were fixed in Bouin's fixative and dehydrated in a graded ethanol series. After an intermediate washing step in acetone, the dissected parts were embedded in araldite epoxy resin. Series of semi-thin sections (1 µm) were made with a rotary microtome (Leica, RM 2165) using either glass or diamond knives. Sections were stained with a mixture of 1% toluidine blue, 1% sodium-tetraborate and 1% pyronin G in an aqueous solution for about 35 s at 60 °C.

Micro-CT

X-ray imaging was performed with a phoenix|x-ray nanotom[®] high resolution micro-computed tomography system (GE Sensing and Inspection Technologies, Wunstorf, Germany) using the proprietary software datos.x acquisition and datos.x reconstruction. Image stacks of virtual sections were exported with the software VG Studio max (Volume Graphics GmbH, Heidelberg, Germany). Some scans were performed using the X-ray Microscope Xradia 410 Versa (Carl Zeiss X-Ray Microscopy, Pleasanton, CA, USA).

3D reconstruction

Every fifth semi-thin section of the series was photographed with a Zeiss AXIO Imager.M1 microscope equipped with a Zeiss AXIOCAM ICc3 camera (Carl Zeiss Microscopy GmbH, Jena, Germany). Digitized sections were aligned automatically and corrected manually with the software Autoaligner 6.0.1. (Bitplane AG, Zurich, Switzerland). Visualizations of both the digitized section and the micro-CT data were performed with the software Imaris (version 6.4. and 7.0, Bitplane AG, Zurich, Switzerland), and Amira 6.4 (Thermo Fisher Scientific, Waltham, MA, USA).

Resin injection

For in-situ vessel depiction and following preparation, casting resins were injected into the heart of specimens of both species. Three different casting resins were applied: (1) PU4ii (vasQtec, Zurich, Switzerland), based on polyurethane, (2) Mercor II (Ladd Research Industries Inc, Williston, VT, USA), and (3) Mercor CL- 2R-5 (SPI-Supplies, West Chester, PA, USA), both based on methyl methacrylate. *F. limosus* specimens were injected with PU4ii while all three resins were used for *P. virginalis* specimens. After curing of the resins, different specimens were either fixed in Bouin's fixative (see “Histology”) for the use in micro-CT or dissolved as corrosion casts following the procedure described in Wirkner and Richter (2004) for scanning electron microscopy.

Scanning electron microscopy

Corrosion casts were sputter coated with gold using a Bal-Tec SCD 004 (duration: 100 s; at the Electron Microscopy Center of the Universität Rostock) and an Emitech, K500 (duration: 100 s; at the Zoological Institute of the FSU Jena). Scanning electron micrographs were taken with FEI Company, XL30 ESEM TMP (at the FSU Jena) and a Zeiss, DSM 960A (at the Universität Rostock).

Image management and processing

Irfan-View 4.0 software was used to convert batches of the digitized images into grayscale, to invert and rename them. CorelDraw Graphics Suite X3 was used to create schematic depictions and to arrange the figure plates.

Terminology

The terminology used in this work is based on the Ontology of the Arthropod Circulatory System (Wirkner et al. 2017). All parts of the circulatory systems are considered morphemes (Richter and Wirkner 2014), i.e. any reference to homology and/or function are explicitly excluded from any of the given descriptions.

Results

In the vascular morphology of *P. virginalis* and *F. limosus*, we found several areas showing intraspecific variability of three different kinds: (1) variability in branching patterns of arterial systems, (2) variable occurrence of anastomoses, and (3) variable asymmetries (Fig. 2). In the following, these three kinds of variability will be exemplified. The gross morphology of the vascular system of both species has been

described in Scholz et al. (2018) and is also summarized in Fig. 1.

Variability in branching patterns

Some artery systems show intraspecific variability in the branching pattern of arteries. This includes different sequence of off-branchings as well as common origins of arteries which are separate in other specimens. In the following, two artery systems, which are subject to a high degree of intraspecific variability in both species, will be described to exemplify this kind of variability (Fig. 2).

Rostro-gastral vasculature

The rostro-gastral vasculature is part of the anterior lateral artery system and supplies the rostral region as well as the stomach. This artery system consists of five main branches (rostral artery, epidermal artery, dorsal artery of the anterior gastric muscle, ventral artery of the anterior gastric muscle, and anterior gastric artery; Fig. 3A). In both crayfish species, the branching pattern is highly variable between the respective specimens and different patterns can be recognized (Fig. 3). In each species, there is a predominant pattern but even in the limited set of specimens investigated in this study (*P. virginalis*: 14 specimens; *F. limosus*: 24 specimens), several alternatives

could be identified (Fig. 3B, C). Although the same five main branches constitute this artery system in both crayfish species, only one of the alternative branching patterns could be identified in both species (Fig. 3B, C).

Posterior aorta system

In both investigated species, the posterior aorta system shows intraspecific variability, for instance in the branching pattern of its distal portion. The terminal bifurcation and the origins of the 4th to 6th posterior lateral arteries show significantly different conditions in different specimens (Fig. 4). The most frequent pattern is the “regular” pattern, with the 4th and 5th posterior lateral arteries emanating separately from the posterior aorta which ultimately bifurcates into the 6th posterior lateral arteries (Fig. 4A). Alternative patterns include a common origin of 5th and 6th posterior lateral arteries (Fig. 4B), a common origin of the 4th and 5th posterior lateral artery on one (Fig. 4C) or both sides (Fig. 4D), asymmetric common origins of two subsequent posterior lateral arteries (Fig. 4E), and the bifurcation of the posterior aorta anterior to the 4th posterior lateral artery (Fig. 4F). In addition to the alternative branching pattern, intersegmental anastomoses (Fig. 4, isan) occur in some specimens (for more on anastomoses, see below).

Fig. 1 Schematic overview of the hemolymph vascular system in the marbled crayfish *P. virginalis* (after Scholz et al. 2018). *aao* anterior aorta, *ala* anterior lateral arteries, *arb*: antenno-renal branch, *ba* brain artery, *cda* caudal artery, *chpa* cardiac hepatic arteries, *dsc* descending artery, *gib* gastro-intestinal branch, *hea* heart, *lb* lateral branch of the anterior lateral artery, *mdb* mandibular branch, *oa* optic artery, *oab* oculo-antennular branch, *os* ostia of the heart, *pao* posterior aorta, *pga1* 1st posterior gastric artery, *plp3* artery of the 3rd pleopod, *ppa2* artery of the 2nd peraiopod, *rgb* rostro gastric branch, *sta* sterna artery, *ta* telson artery, *upd* uropodal arteries

Fig. 2 Schematic overview of regions of the hemolymph vascular system showing intraspecific variability (lateral view). **A** Regions of the HVS showing variable branching patterns; filled circles show regions that are explained in the text. i: rostral-gastral vasculature; ii: terminal bifurcation of the posterior aorta; iii: branching pattern of the posterior gastric artery system; iv: course, number and caliber of the descending arteries; v: number of cardiac hepatic arteries; vi: junction pattern of arteries of the mouthparts in the anterior part of

the ventral vessel system; vii: junction pattern of thoracopodal arteries in the ventral vessel system; viii: aortic bulb region. **B** Regions of anastomoses with intraspecific variability; *anlan* antennular anastomosis, *apan* anterior pyloric anastomosis, *cdan* caudal anastomosis, *ean* esophageal anastomosis, *isan* intersegmental anastomosis between neighboring posterior lateral arteries, *roan* rostral anastomosis, *sgan* subgastric anastomosis, *tlan* telsonic anastomosis between uropodal arteries

Fig. 3 Schematic illustration of the rostro-gastral vasculature. **A** Overview, from lateral, of the distal branching pattern in relation to the rostral apodeme (rap) and the anterior gastric muscle (agm) in *P. virginalis*. The five main arteries, i.e., rostral artery (roa=A), epidermal artery (ea=B), dorsal artery of the anterior gastric muscle (agd=C) and ventral artery of the same (agv=D) and the anterior

gastric artery (aga=E). **B - C** Schemes in lateral view of the most frequent branching patterns. The intraspecifically varying regions are marked in black. **B** Pattern in *P. virginalis* and **C** in *F. limosus*. Below the drawings, the observed branching formulas are listed. The underline marks the only pattern occurring in both species. Brackets indicate arteries emanating from a common branch

Fig. 4 Schematic illustration of the alternative branching patterns of the distal portion of the posterior aorta system. isan: intersegmental anastomosis; pao: posterior aorta; pla4–6: 4th to 6th posterior lateral artery

Anastomoses

Anastomoses, i.e. connecting arteries bypassing two artery systems, occur in numerous regions of the hemolymph vascular system in both crayfish species (Fig. 2B). These anastomoses, however, are not present in each single specimen but the occurrence rather shows remarkable intraspecific variability. In the following, variably occurring anastomoses will be described for exemplar regions of the crayfish vascular systems.

Gastro-intestinal artery system

The gastro-intestinal artery is part of the anterior lateral artery system. It runs medially and ramifies to form the gastro-intestinal artery system which supplies the stomach and the anterior midgut. The gastro-intestinal artery system of each side consists of three main branches, the three posterior gastric arteries (Fig. 5, pga1–3). In the ventral median line, right beneath the stomach, the subgastric artery runs longitudinally and is connected to the first posterior gastric artery via one or more anastomoses. In the marbled crayfish, the subgastric artery anteriorly sends off an anastomosis to connect with the sternal artery (Fig. 5A). In both species, intraspecific variability shows in the irregular occurrence of anastomoses between the anterior and posterior branch of the 1st posterior gastric artery of either side and the subgastric artery (Fig. 5B, C).

Posterior aorta system

As mentioned above, the posterior aorta system not only shows intraspecific variability in the branching pattern of its distal region but also in the occurrence of intersegmental anastomoses (Figs. 2, 3). These anastomoses connect the

posterior lateral arteries of neighboring pleomeres and can occur independently on both sides (Fig. 4). Between both extremes, i.e. no intersegmental anastomoses on the one hand and intersegmental anastomoses between all posterior lateral arteries forming two continuous parallels to the posterior aorta on the other hand, all graduations might occur as numerous patterns of intersegmental anastomoses were observed in both species.

Variable asymmetries

In the crayfish circulatory systems, asymmetries in vascular morphology are a frequent phenomenon. While some such asymmetries are the result of variable branching patterns (see above; Fig. 4), other asymmetries are based on the unilateral occurrence of entire artery systems or variable unilateral development.

Descending artery

The descending artery of *P. virginalis* is an unpaired artery in most specimens (see Table 1 for numbers in both species).

This artery passes the midgut either on the right or left side (as already described by Vogt et al. 2009). In some specimens, however, the descending artery is paired. In this case, both descending arteries can either be equally strong or one of the two arteries is stronger.

Hepatic artery system

Another remarkable case of asymmetry was found in the hepatic artery system of *P. virginalis*. A single cast of 25 observable ones of the hepatic artery showed only the left hepatic artery present supplying the entire midgut gland. In this specimen, the left hepatic artery gives rise to a

Fig. 5 Schematic illustration of anastomoses of the gastro-intestinal artery system (pga1). **A** Overview of the situation in lateral view, casts of 17 specimens of both, *P. virginialis* and *F. limosus* could be observed. The posterior gastric artery system with its three main branches (pga1–3) and the gastric mill (green) with the esophagus (es), gastrolith (gl) and pylorus (pyl). The posteriormost side branch (pga1a) and the subgastric artery (sga) supply the midgut gland via auxiliary hepatic vessels (ahv). The anastomosis between sternal artery (sta) and sga via median esophageal vessel (mev) was only found in *P. virginialis*; **B–H** Schematic representations of the variations of the branching pattern found in the observed species, depicted in a ventral view; black are varying parts, grey is the sga in variations occurring in both species because its anterior section is absent in *F. limosus*; **B** Anastomosis of pga1b anterior from the pylorus is the most common variation in *P. virginialis* ($n=7$); **D** Most common variation in *F. limosus*

prominent branch crossing ventrally of the midgut to the right side, where it runs dorsally and ramifies into an elaborate vasculature supplying the entire right side of the midgut gland (Fig. 6).

Discussion

Although the development of arteries in crustaceans is not readily understood (Wirkner et al. 2013), intraspecific variability has also been reported for other arthropods such as other decapods (e.g. Keiler et al. 2013), stomatopods (unpublished data), horseshoe crabs (Göpel and Wirkner 2015), various arachnids (e.g. Klussmann-Fricke et al. 2012; Runge and Wirkner 2016; Huckstorf and Wirkner 2019) and chilopods (unpublished data). Our data show that distinct

parts of the vascular morphology of crayfish are subject to non-genetic phenotypic variation.

In general, intraspecific variability in vascular morphology described for *P. virginialis* can either be ascribed to phenotypic plasticity or stochastic developmental variation due to their genetic identity (Martin et al. 2007; Gutekunst et al. 2018). Since all individuals of *P. virginialis* were kept under identical conditions, phenotypic plasticity induced by external environmental factors appears unlikely at first. Thus, a possible explanation for this non-genetic phenotypic variation is stochastic developmental variation as result of stochastic alterations of the epigenome (Vogt 2015, 2020). We were able to find congruent phenomena of intraspecific variability in the sexually reproducing crayfish *F. limosus*. Furthermore, Baumann (1921) described the same phenomena of intraspecific variability for *Astacus astacus*. Due to this congruence in variable patterns across all three species

Table 1 In the table the number of specimens is depicted showing the respective vascular structures in accordance to the chosen method

Artery system	Vascular structure	Species	Specimen data			Symmetry states				Variation states		Methods
			Determinable	Present	Absent	Unilateral	Only right	Only left	Bilateral	Symmetric	Asymmetric	
Anterior aorta	Proximal side branches	Pv	26	2	24							38 cc
		Ol	22	0	22							24 cc
Anterior lateral artery	Antennular anastomosis	Pv	10	10								38 cc
		Ol	13	13								24 cc
	Anterior pyloric anastomosis	Pv	17	8	9							38 cc
		Ol	17	4	8							24 cc
	Posterior pyloric anastomosis	Pv	17	1	15							38 cc
		Ol	17	0								24 cc
	Accessory hepatic arteries	Pv	18	17								38 cc
		Ol	17	11	6							24 cc
	Distal branching pattern of rostro-gastral branch	Pv	14								6	38 cc
		Ol	24									24 cc
	Anterior pyloric artery in subgastric artery	Pv	17	14		5	2	3	9	9		38 cc
		Ol	17	16		12	6	6	4	4		24 cc
	Posterior pyloric artery in subgastric artery	Pv	17	3		2	1	1	1		3	38 cc
		Ol	17	2		2	2				2	24 cc
Hepatic artery	One supplying both sides	Pv	25	1	24	1		1	24			38 cc
		Ol	22	0	22							24 cc
Descending artery	Symmetry	Pv	53	53		31	13	18	16	4	1R, 11L	14 sss; 38 cc; 1 pct
		Ol	12	12		12	5	7			2	24 cc
	Side-branches	Pv	18	15	3							38 cc; 1 sss
		Ol	17	17								24 cc
Ventral vessel	Esophageal anastomosis	Pv	7	4	3							38 cc
		Ol	10	1	9							24 cc
	Subgastral anastomosis	Pv	31	11	20							38 cc

Table 1 (continued)

Artery system	Vascular structure	Species	Specimen data		Symmetry states			Variation states		Methods
			Determinable	Present	Absent	Unilateral	Only right	Only left	Bilateral	
		Ol	24	0						24 cc
	Origin of the 2nd thoracopod artery: separately	Pv	17	17						38 cc
		Ol	24	12						24 cc
	On th1a I	Ol	24	2						24 cc
	Next to th1a I	Ol	24	3						24 cc

cc corrosion cast, μ CT micro-computed tomography, sss serial semi-thin sections

and the genetic uniformity of *P. virginalis*, the intraspecific variability in vascular morphology in the two sexually reproducing crayfish species is most likely to result from the same mechanism.

The numerous regions subject to phenotypic variation in the crayfish vasculature lead to the assumption that mainly the major arteries are genetically determined while most ramifications around other organs are not. The obvious relevance of non-genetic phenotypical variation in vascular morphology bears a significant impact on the interpretation of vascular morphology in terms of putative evolutionary scenarios. Furthermore, such findings on the high prevalence of non-genetic phenotypic variation in morphology need to be considered for evolutionary morphology and the conceptualization of morphological characters in general.

Stochastic developmental variation or phenotypic plasticity due to microenvironments?

Since phenotypic plasticity and stochastic developmental variation represent complementary aspects of non-genetic phenotypic variation (Furness et al. 2015; Leung et al. 2016), it makes sense to not assess their evolutionary importance as isolated mechanisms but as two aspects of one and the same general phenomenon, i.e. genetic indeterminacy of phenotype development. Although mostly discussed in the light of phenotypic plasticity, suggested evolutionary advantages hold true for non-genetic phenotypic variation in general in due consideration of plasticity and stochastic variation as complementary mechanisms (Simons 2014; Leung et al. 2016). Non-genetic phenotypic variation allows populations to face environmental change in shorter time than genotype evolution could possibly do (Burggren 2018; Angers et al. 2020). Thus, non-genetic phenotypic variation appears evolutionary favorable in variable environments (Hendry 2016; Burggren 2018) with phenotypic plasticity being more frequent in environments with rather predictable change and stochastic developmental variation in unpredictable environments (Furness et al. 2015; Leung et al. 2016).

Stochastic developmental variation produces different phenotypes as result of epigenetic variation (Vogt 2017, 2020; Angers et al. 2020), whereas environmentally induced phenotypes can actually be manifested into the epigenome (Bautista et al. 2020). As transgenerational phenotypic plasticity due to epigenetic inheritance has been shown in several studies (e.g. Holeski et al. 2012; Serobyan and Sommer 2017; Schmid et al. 2018; Bautista and Burggren 2019; Bautista et al. 2020), it is just as plausible to assume such epigenetic inheritance (via inherited methylation patterns, histone modifications, etc.; Holeski et al. 2012; Schmid et al. 2018; Bautista et al. 2020) for phenotypes resulting from stochastic developmental variation. As some phenotypes produced in such a bet-hedging strategy will show a higher

Fig. 6 Unpaired hepatic artery in a specimen of *P. virginalis*. **A** Photograph of a corrosion cast in anterior view. **B** SEM of the same corrosion cast in ventral view. *ahb* anterior hepatic branch, *hepa* cardiac hepatic artery, *mhb* mid hepatic branch; *phb* posterior hepatic branch

fitness than others will, frequency of the epialleles will likely be shifted in the next generation. Whether such inherited epialleles become directly incorporated in the genome, however, is yet to be shown on a broader scale. While there are single indications of such direct mechanisms (e.g. Skinner et al. 2015), other indirect effects of epigenetic inheritance on selection and thus evolution are discussed (see Burggren 2016).

A putative selection towards a certain degree of indeterminacy, leading to phenotypic plasticity and stochastic developmental variation, is thus easily assumed, however, appears difficult to be scientifically proven (Via 1993; Pigliucci 2005).

Since mother individuals of the marbled crayfish were kept here under the same conditions, phenotypic plasticity due to the general environmental factors in the tank (e.g. temperature, oxygen saturation, pH, photoperiod) would have to be excluded at first glance. Does this leave stochastic developmental variation (Vogt 2015, 2020) as the only possible explanation for such non-genetic phenotypic variation? Stochastic developmental variation can be ascribed to random epigenetic alterations having an impact on developmental processes (Leung et al. 2016; Angers et al. 2020). The phenotypic variation studied herein might (at least in part) be the result of stochastic developmental variation. However, we think that phenotypic plasticity cannot be excluded completely. While the general conditions in the tank might have been identical for the mother individuals, microenvironments for the developing embryos certainly differ within each clutch. In egg clutches of fishes, position of the individual egg in the egg mass correlates with phenotypic differences (size, body mass, metabolic rate) between the siblings

(Green et al. 2006; Burton et al. 2013). Egg clutches obstruct water flow, which leads to differences in the water chemistry (e.g. oxygen saturation, pH) for eggs located within the egg mass (Dhiyebi et al. 2013). These flow-related crowding effects occur already due to mere presence of a single layer of neighboring eggs and are further amplified by their metabolism (Dhiyebi et al. 2013). Such effects may well lead to developmental responses in form of phenotypic plasticity leading to different phenotypes of the offspring as result of different microenvironmental conditions during development. It is thus plausible to assume that the differences, e.g. in oxygen supply, lead to different physiological necessities for the developing organs in the crayfish embryo. Different vascular patterns, a regionally more extensive vasculature or certain anastomoses, as found in this study, might optimize supply for certain developing tissues and organs and might form as result of phenotypic plasticity induced by these microenvironmental conditions.

Characterization of the epigenome alone will also not be able to provide a definitive answer to the question of the origin of the different phenotypes. While phenotypic variation due to stochastic developmental variation follows random epimutations (Angers et al. 2020), phenotypic plasticity, in turn, can lead to manifestation of the environmentally induced phenotypes into the epigenome and even allow for transgenerational epigenetic inheritance of plasticity induced phenotypes (Bautista et al. 2020).

To answer this question, artificial incubation of the eggs with guaranteed identical environmental conditions for each egg needs to be performed. To further clarify, whether the phenotype follows the epigenome or vice versa, methylation patterns, histone modifications etc. would need to be

compared at the beginning of incubation and after hatching. This, however, appears hardly possible in early embryos without at least altering (in fact rather damaging or sacrificing) them, as epigenetic profiles differ between tissues (Leung et al. 2016) and thus early somatic cells from the embryo would be necessary. Novel strategies to differentially assess phenotypic plasticity and stochastic developmental variation thus need to be developed.

Non-genetic phenotypic variation in evolutionary morphology

In evolutionary morphology, characters are conceptualized to infer phylogenetic hypotheses and/or reconstruct events of evolutionary transformation (Wirkner and Richter 2010; Richter and Wirkner 2014). Characters represent hypotheses on units of evolution and thus on subjects of selection and adaptation (Wagner 2000, 2014; Richter and Wirkner 2014). Biological individuality and inheritability are considered prerequisites for entities to constitute a character (Wagner 2014). Such morphemes, (i.e. observable physical constituents of organisms; Richter and Wirkner 2014), which are subject to either phenotypic plasticity or stochastic developmental variation are not genetically determined and thus not inheritable in that sense across numerous generations (as opposed to inherited epigenetic variations which generally dissipate over a few generations or even over successive broods of the same generation; Burggren 2015) which means they cannot represent an evolutionary character (Wagner 2000, 2014; Richter and Wirkner 2014; Göpel and Richter 2016). For instance, the branching pattern of the distal portion of the posterior aorta system, cannot be a character in an analysis of crayfish as none of the actually observed patterns is genetically determined and thus inheritable. The fact that a certain morpheme is subject to phenotypic plasticity or stochastic developmental variation, however, can be a character itself in the sense that a lack of genetic determination of a certain morphological pattern in some taxa (as opposed to certain other taxa with genetic determination of said morphological pattern) might be treated analogous to the character state *absent* in neomorphic characters (Serenio 2007). Non-genetic phenotypic variation must therefore be carefully considered in the context of evolutionary morphology and a sufficient number of individuals need to be investigated in order to conceptualize morphological characters.

Conclusion

Morphology of the hemolymph vascular system in crayfish shows a significant degree of non-genetic phenotypic variation in terms of varying branching patterns, asymmetries and variably occurring anastomoses. Because of its relevance as

supplying organ system in the integral organism, morphology of the circulatory system as well as cardiorespiratory physiology appear worthwhile investigating in terms of both phenotypic plasticity and stochastic developmental variation as response to fast environmental changes (e.g. Leung et al. 2016; Burggren 2018). The marbled crayfish *P. virginalis* has proven suitable as object of study in terms of stochastic developmental variation as well as phenotypic plasticity (Vogt 2020). Future work might include investigation of cause and effects regarding variation in vascular morphology as well as the impact of non-genetic phenotypic variation of cardiorespiratory physiology.

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