

MORPHOLOGICAL INTEGRATION AND ALTERNATIVE LIFE HISTORY STRATEGIES:
A CASE STUDY IN A FACULTATIVELY PAEDOMORPHIC NEWT

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ABSTRACT

Tetrapod limbs are serially homologous structures that represent a particularly interesting model for studies on morphological integration, i.e. the tendency of developmental systems to produce correlated variation. In newts, limbs develop at an early larval stage and grow continuously, including after the habitat transition from water to land following metamorphosis. However, aquatic and terrestrial environments impose different constraints and locomotor modes that could affect patterns of morphological integration and evolvability. We hypothesize that this would be the case for alternative heterochronic morphs in newts, i.e. aquatic paedomorphs that keep gills at the adult stage and adult metamorphs that are able to disperse on land. To this end, we analysed patterns and strengths of correlations between homologous skeletal elements of the fore- and hindlimbs as well as among skeletal elements within limbs in both phenotypes in the alpine newt, *Ichthyosaura alpestris*. Our results showed that metamorphs and paedomorphs had similar, general patterns of limb integration. Partial correlations between homologous limb elements and within limb elements were higher in paedomorphs when compared to metamorphs. Decrease in partial correlation between homologous limb elements in metamorphs is accompanied with a higher evolvability of the terrestrial morph. All these results indicate that environmental demands shaped the patterns of morphological integration of alpine newt limbs and that the observed diversity in correlation structure could be related to a qualitative difference in the modes of locomotion between the morphs.

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INTRODUCTION

Morphological integration (Olson and Miller, '58), the correlation of phenotypic traits in relation to their genetic, functional and developmental relationships, has been in the scope of evolutionary biology research for several decades (Cheverud, '82; Hallgrímson et al., 2002; Goswami, 2006; Klingenberg, 2008; Porto et al., 2009). It is recognized to have important implications for the evolutionary potential of morphological traits through the structuring of phenotypic variation (Hallgrímson et al., 2009). Various covariance-generating processes could influence the structure and pattern of morphological integration, including environmental (Fischer-Rousseau et al., 2009) or functional demands (Martin-Serra et al., 2015). These processes could overprint the covariance pattern produced by common genetic background or developmental pathways, making the individual sources of covariance and their interactive effects hard to decipher (Hallgrímsson et al., 2009; Labonne et al., 2014; Klingenberg, 2014).

Serially homologous structures represent particularly interesting systems for studies of morphological integration due to the strong impact of development on their covariation (Young and Hallgrímson, 2005). In tetrapods, limbs represent serially homologous structures (for a different view, see Diogo et al., 2013; Diogo and Ziermann, 2014) in which the developmental properties are duplicated and expressed in a new location and time (Hall, '95). Fore- and hindlimbs have the same three basic anatomical segment structures: first, the stylopod (humerus and femur), second, the zeugopod (radius/ulna, tibia/fibula) and third, the autopod (carpals/metacarpals, tarsals/metatarsals, phalanges). They also share similar genetic and developmental pathways (Margulies et al., 2001; Shou et al., 2005; Hallgrímsson et al., 2009; Young et al., 2010; Petit et al., 2017). Because of their shared developmental, functional and/or genetic influences, the fore- and hindlimbs could therefore be considered as highly integrated (Olson and Miller, '58; Kelly and Sears, 2011). Empirical studies found that different functional demands on fore- and hindlimbs can influence developmental integration (Kelly and Sears, 2011; Bell et al., 2011). Also, independent specialization of forelimbs and hindlimbs could be achieved by evolutionary change in the integration pattern and dissociation in the correlation pattern between fore- and hindlimbs. This has been exemplified in the evolution of various groups including hominids (Hallgrímsson et al., 2009; Rolian, 2009; Young et al., 2010), marsupials (Kelly and Sears, 2011) and three clades of flying vertebrates (Bell et al., 2011).

In tailed amphibians, fore- and hindlimbs have a similar basic morphological organisation and functional demands in locomotion. Limbs form at an early larval stage and develop continuously after hatching. Free-living larvae move, using limbs as they develop. The limbs are completely developed at late larval stage, according to Glücksohn's staging table at stage 62 (Glücksohn, '32). The development and morphology of salamander's limbs is well studied (Shubin and Alberch, '86; Blanco and Alberch, '92; Vorobyeva and Hinchliffe, '96). Although it is well known that thyroxine, "the metamorphic hormone in amphibians" (Hall, 2005), affects ossification and development of appendicular skeleton in anurans, the development of appendicular skeleton in salamandrid salamanders is considered to be thyroxine-independent (Smirnov and Vassilieva, 2003). However, some effects of exogenous thyroxine on the deposition calcified matrix were

recorded (Smirnov and Vassilieva, 2003). The environmental shift from water to land during the process of metamorphosis imposes different demands for locomotion (Gvoždik and Van Damme, 2006). Locomotion in larvae is aquatic, primarily characterized by passive buoyancy and moving by beats of the tail. After metamorphosis, newts and salamanders leave the water for land where locomotion creates different bone loading patterns, as limbs must be able to support the weight of the body and provide forward propulsion. Locomotion on land is accomplished by synchronous use of diagonal limb pairs, whereas for locomotion in water the limbs are held tightly against the body to reduce hydrodynamic drag during swimming (Azizi and Horton, 2004). This ontogenetic niche shift coupled with increased calcification during metamorphosis (Smirnov and Vassilieva, 2003) could affect the pattern of morphological integration of limb elements across life stages.

An alternative to the usual metamorphosis process is paedomorphosis. It is defined as an evolutionary heterochronic phenomenon when adult organisms retain larval characteristics (Gould, '77; Laudet, 2011; McNamara, 2012). In facultative paedomorphic populations of tailed amphibians, environmentally-induced polymorphism results in the coexistence of two different morphs: gilled and fully aquatic paedomorphic adults and transformed terrestrial metamorphic adults in the same population (Semlitsch and Wilbur, '89; Whiteman, '94; Denoël et al., 2005; Oromi et al. 2016). Metamorphic individuals walk on land and return to the aquatic habitat during the reproductive period, while paedomorphic individuals remain in water. Several species, such as the alpine newt have been reported to live in deep lakes where paedomorphs can use the water column predominantly, a micro-habitat where the main locomotor pattern is swimming (Denoël and Joly, 2001). It has been shown that facultative paedomorphosis can influence morphometric variation between morphs. Paedomorphs and metamorphs diverge in body size (Wilbur and Collins, '73; Džukić et al., '90; Kalezić et al., '96), sexual dimorphism (Denoël et al., 2009) and overall integration of the cranial and postcranial skeleton (Ivanović et al., 2005). However, the changes in the pattern of covariance and possible sources that influence covariance across ontogenetic stages remain largely unexplored. By using facultative paedomorphosis as a developmental model system, we aimed to explore how divergence in ontogeny (metamorphosis versus paedomorphosis) and different biomechanical requirements (primary need for terrestrial walking versus swimming and aquatic walking) affect the correlation structure of limbs. To this end, we analysed the correlation structure of limb skeletal elements of paedomorphic and metamorphic individuals to determine the extent of developmental and functional influences on integration and evolvability. We focused on four skeletal elements of each limb to estimate correlation patterns as a function of different ontogenetic trajectories and functional demands. If developmental constraints determine the patterns of limb integration, we would expect high correlations between homologous limb elements and no differences between morphs. Also, if selection related to different locomotor modes has little or no effect, there should be no difference in evolvability between two morphs. However, if sufficient variation in the developmental processes regulating limb length exists, then changes in ontogenetic trajectories or the locomotion mode (from only swimming to walking and swimming) could alter patterns of fore- to hindlimb integration and ability of the limbs to respond to selection (evolvability).

MATERIALS AND METHODS

Species and sample

The alpine newt, *I. alpestris*, is the only living representative of the genus *Ichthyosaura* (Recuero et al., 2014), with a relatively long independent evolutionary history (42.4 million years, Arntzen et al., 2015). The genus *Ichthyosaura* is closely related to the clade of small bodied newts (genus *Lissotriton*) within the large clade of Modern Eurasian newts (sensu Steinfartz et al., 2007). Both clades are characterised by the evolution of facultative paedomorphosis in a number of populations. We analysed individuals from three populations of the alpine newt, all with a high incidence of paedomorphosis at the time of sampling (Kalezić and Džukić, '86; Kalezić et al., '96; Denoël and Schabetsberger, 2003; Denoël et al., 2004). Two populations were from Montenegro and one from Greece (Table 1). All analysed specimens were adults with a developed cloaca and fully mature gonads (see also Denoël, 2017). Males differ from females by the shape of the cloaca as well as other dimorphic traits. Paedomorphs differ from metamorphs by larval characters, such as the presence of external gills.

The cleared and stained skeletons (for bone with alizarin red and cartilage with alcian blue, following Dingerkus and Uhler ('77)) (for the detailed procedure see Tomašević Kolarov et al., 2011) were stored in the Batrachological Collection of the Institute for Biological Research "Siniša Stanković", Belgrade, Serbia (Džukić et al., 2015). For collection numbers see supplementary Table S1.

Table 1. Studied populations of *I. alpestris*, geographic data and sample sizes of metamorphs and paedomorphs per population. MNE – Montenegro, GR – Greece.

Population	Coordinates with altitude	Metamorphs		Paedomorphs	
		Females	Males	Females	Males
Bukumirsko lake, MNE	42°36'N, 19°33'E (1,446 m)	9	10	12	13
Manito lake, MNE	42°48'N, 19°14'E (1,773 m)	7	10	6	6
Drakolimni, GR	39°59'N, 20°47'E (2,140 m)	11	7	15	14

Data collection

Limbs skeletal elements were photographed with a scale bar (10 mm) using a Moticam 2000 camera connected to a Nikon SMZ800 stereo-zoom microscope. Two-dimensional digital landmarks of maximal length of four skeletal elements in both the forelimbs (H - humerus, R - radius, MC - metacarpal of the third digit, Ph 3.1- first phalanx of the third digit) and hindlimbs (F-femur, T-tibia, MT-metatarsal of the third digit, hPh 3.1- hindlimb first phalanx of the third digit) (Fig. 1) were digitized using TpsDig software (Rohlf, 2005). The dimensions of skeletal elements (in mm) were calculated as Euclidian distances between the given landmarks using IMP software (Sheets, 2000). Data were collected from right fore- and hindlimbs. Mean values and standard deviations of analysed skeletal elements in metamorphs and paedomorphs of *I. alpestris* given by sex and population are shown in Table 2.

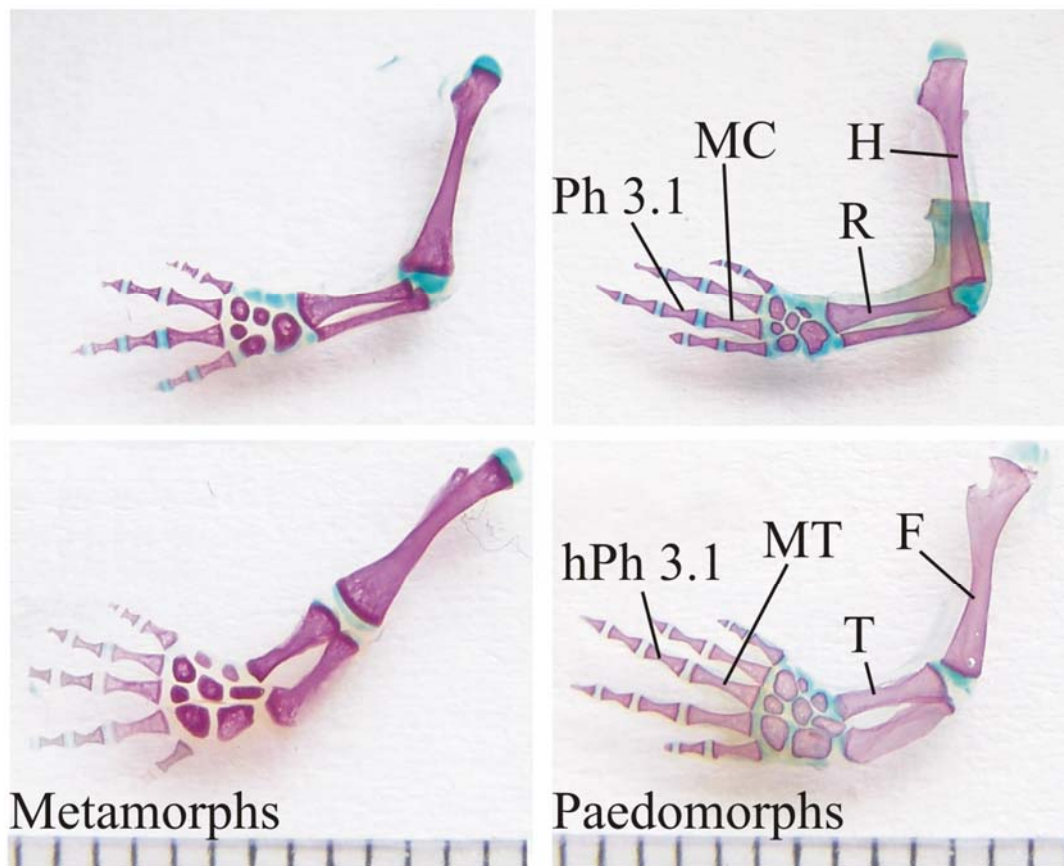


Figure 1. The right fore- and hindlimb of a paedomorphic and metamorphic *I. alpestris* with the skeletal elements used in the analysis: Forelimb: H - humerus, R - radius, MC - metacarpal of the third digit, Ph 3.1- first phalanx of the third digit; Hindlimb: F - femur, T - tibia, MT - metatarsal of the third digit, hPh 3.1- hindlimb first phalanx of the third digit.

Table 2. Mean values (with standard deviations given in parenthesis) of analysed skeletal elements in metamorphs (A) and paedomorphs (B) of *I. alpestris* given by sex and population. Forelimbs (H - humerus, R - radius, MC - metacarpal of the third digit, Ph 3.1- first phalanx of the third digit) and hindlimbs (F - femur, T - tibia, MT - metatarsal of the third digit, hPh 3.1- hindlimb first phalanx of the third digit). F - females, m - males; N -number of individuals; Total - total number of metamorphs and paedomorphs; GM – log transformed geometric mean; CV – coefficient of variation.

A) Metamorph			H	R	MC	Ph3.1	F	T	MT	hPh 3.1
population	sex	N								
Bukumirsko lake	f	9	6.33 (0.29)	3.68 (0.22)	1.66 (0.12)	0.97 (0.08)	5.99 (0.28)	3.21 (0.20)	1.91 (0.13)	1.08 (0.08)
	m	10	5.88 (0.31)	3.3 (0.20)	1.62 (0.13)	0.95 (0.13)	5.54 (0.29)	2.96 (0.16)	1.92 (0.12)	1.17 (0.08)
Manito lake	f	7	6.37 (0.48)	3.45 (0.28)	1.66 (0.15)	0.98 (0.11)	5.91 (0.42)	3.2 (0.33)	1.86 (0.19)	1.07 (0.08)
	m	10	5.78 (0.31)	3.25 (0.19)	1.57 (0.10)	0.95 (0.09)	5.52 (0.30)	2.93 (0.18)	1.82 (0.19)	1.09 (0.14)
Drakolimni	f	11	5.73 (0.30)	3.15 (0.15)	1.42 (0.14)	0.83 (0.13)	5.59 (0.23)	2.73 (0.13)	1.66 (0.15)	0.95 (0.11)
	m	7	5.55 (0.33)	3.05 (0.16)	1.52 (0.11)	0.96 (0.08)	5.25 (0.26)	2.65 (0.13)	1.79 (0.15)	1.21 (0.11)
Total		54	GM=0.403 (CV=0.060)							
B) Paedomorph			H	R	MC	Ph3.1	F	T	MT	hPh 3.1
population	sex	N								
Bukumirsko lake	f	12	6.32 (0.41)	3.62 (0.28)	1.61 (0.17)	0.9 (0.12)	5.92 (0.36)	2.99 (0.22)	1.82 (0.16)	1.01 (0.13)
	m	13	5.83 (0.48)	3.36 (0.30)	1.58 (0.16)	0.92 (0.14)	5.33 (0.49)	2.78 (0.28)	1.79 (0.19)	1.13 (0.16)
Manito lake	f	6	6.26 (0.46)	3.47 (0.28)	1.51 (0.13)	0.84 (0.17)	5.83 (0.45)	3 (0.17)	1.62 (0.11)	0.91 (0.09)
	m	6	5.87 (0.40)	3.17 (0.24)	1.44 (0.12)	0.81 (0.12)	5.5 (0.34)	2.79 (0.19)	1.72 (0.11)	1.03 (0.14)
Drakolimni	f	15	5.42 (0.29)	3.09 (0.18)	1.4 (0.14)	0.85 (0.09)	5.32 (0.25)	2.63 (0.16)	1.68 (0.12)	1 (0.10)
	m	14	5.28 (0.18)	2.95 (0.09)	1.43 (0.10)	0.91 (0.10)	5.15 (0.11)	2.51 (0.09)	1.72 (0.11)	1.1 (0.12)
Total		66	GM=0.385 (CV=0.074)							

Data adjustments

The fore- and hindlimb data were used to estimate limb phenotypic correlation matrices for each morph. Analyses of morphological integration are highly sensitive to different types of variation in the data (sexual dimorphism in size, population structuring, small sample size) (Goswami and Polly, 2010). Therefore, raw data were adjusted to reduce variation not relevant to this study. First, data from the two sexes were pooled within each population/morph after adding the mean differences between males and females to the sex with the smaller mean (Marroig and Cheverud, 2004). Second, the same approach was also used to pool populations within each morph (adding the difference in means between the two populations to the population with the smaller mean).

Since differences in body size and size-related variation can also influence integration patterns and evolvability (Marroig and Cheverud, 2004; Rolian, 2009; Lewton, 2012), we applied a normalization method to scale data and remove allometric effects (Lleonart et al., 2000). The geometric mean (GM of 8 limb elements) was used as an overall measure of size. For each morph, average GM was calculated and log-transformed. Also, each limb element was log-transformed and regressed onto average GM (morph-specific). The slopes of each regression were used to adjust individual limb elements following equation from Lleonart et al. (2000): $Y_i^* = Y_i [X_0 / X_i]^b$, where Y_i^* is the theoretical value of a trait given the group average size for individual i , Y_i and X_i are the values of the specific trait and overall size (GM) for individual i , respectively, X_0 is the group average size (average GM) and b is the coefficient of allometry for each trait (Marroig and Cheverud, 2004; Rolian, 2009; Lewton, 2012). To compare morphs, allometrically adjusted data for each individual were then adjusted by its average size, scaling all individuals in both morphs to a theoretical body size of 1. Both raw (after the sex and population adjustments) and size-corrected data were used to derive phenotypic correlation/variance-covariance matrices.

Patterns of correlation

We applied multiple methods to investigate patterns of correlation because they were used successfully in previous studies to advance our understanding of the degree to which patterns of morphological integration are conserved. First, correlation matrix repeatability was assessed with a Monte Carlo simulation (1000 replicates) to estimate the impact of sampling error. The original dataset was resampled with replacement and the correlation matrices were re-estimated 1000 times (Marroig and Cheverud, 2001). Then, morph-specific correlation matrices for three data sets (all limb elements, only forelimb elements and only hindlimb elements), were generated using Pearson's product moment correlation coefficient. These correlation matrices were subjected to matrix correlation analysis and Mantel's test to show whether the matrices were more similar to each other or to randomly generated matrices. Significance of the matrix correlations was confirmed when the observed matrix correlation exceeded 95% of the randomly generated correlations. Third, patterns of limb correlation were examined separately for metamorphic and

paedomorphic newts using partial correlation analysis (full 8 variable matrix) with the edge exclusion deviance statistic (Magwene, 2001), as a significance test for conditional independence of limb elements. Partial correlations measure associations between two variables that are independent of information from other variables in the correlation matrix. The significance of partial correlations was assessed, using an information theoretic approach known as the edge exclusion deviance (EED) and the χ^2 distribution: $EED = -N \ln(1 - \rho^2_{ij(K)})$, where N is the sample size and $\rho^2_{ij(K)}$ is the partial correlation coefficient between variables i and j (Magwene, 2001). Two variables are conditionally independent when the EED value is less than 3.84 (corresponding to $p = 0.05$, $df = 1$ from the χ^2 distribution). For partial correlation analysis, we used only the raw dataset following the recommendations of Magwene (2001), as conditioning the elements of a partial correlation matrix on every other variable, implicitly estimates size (Whitaker, '90; Lawler, 2008). We calculated mean within limb (for fore- and hindlimbs) and mean between limb (all between limb elements and only homologous limb elements) absolute partial correlations for both paedomorphs and metamorphs. Also, partial correlation matrices were subjected to matrix correlation analysis and Mantel's test to determine whether the matrices were structurally more similar to each other or to randomly generated matrices. Significance of the matrix correlations was confirmed when the observed matrix correlation exceeded 95% of the randomly generated correlations. Fourth, the degree of overall correlation of metamorphic and paedomorphic newt limbs was estimated by the index of integration, which was calculated as the variance of eigenvalues (VE) (Wagner, '84). Eigenvalue variance of the correlation matrix was standardized by the maximum possible eigenvalue variance to allow comparison between morphs as they may differ in size (Pavlicev et al., 2009): $VE_{SD} = VE / N - 1$, where VE_{SD} is the standardized eigenvalue variance, VE is the observed eigenvalue variance and N is number of traits in the correlation matrix. Higher correlation among traits corresponds to higher values of VE and *vice versa*. The significance of differences in eigenvalues between morphs was calculated by resampling the data with replacement and re-computing the VE (Manly, '91). The p -value was obtained as the number of times the VE in the morph with smaller VE exceeds the bootstrapped values in the morph with larger VE , divided by the number of iterations (i.e., 1000). All calculations were made using PopTools 2.62, CSIRO, Canberra (Hood, 2004) and selection and response vectors were performed with the `matrix` and `lapply` functions in the base R package (R Development Core Team 2015).

Evolvability

The ability of the limbs to respond to selection were evaluated via methods proposed by Hansen and Houle (2008) which are derived from Lande's ('79) multivariate selection equation: $\Delta z = G\beta$, where Δz is the response vector, G is the genetic covariance matrix and β is a selection vector (the vector of partial regression coefficients of the traits on fitness). Here the phenotypic covariance matrix P is substituted for G , due to lack of genetic covariances for this dataset. Several studies have shown that phenotypic and genetic covariance matrices are proportional and similarly structured (Cheverud, '88; but see Willis et al., '91).

The covariance matrix for each morph was subjected to 1000 randomly generated selection vectors and the angle between selection and response vectors was calculated for each iteration. The mean cosine of the 1000 angles between selection and response vectors is the mean evolvability for each morph. Mean evolvability describes the degree to which the response and selection vectors are aligned in multivariate space. The values of evolvability close to 1 corresponds to high respond of population mean in all directions that selection pushes it in phenotypic space. Evolvability close to 0 implies more constrained variation as the traits became more integrated. The evolvability index was calculated for raw and scaled data. Statistical significance of the differences in the evolvability index was assessed by the same resampling approach as in the VE method.

RESULTS

Correlation matrix repeatability was high, with values of 0.95 (0.99 for scaled data) for metamorphic newts and 0.93 (0.98 for scaled data) for paedomorphic newts, indicating a robust data set for both morphs in the alpine newt. Matrix correlation analysis showed that metamorphs and paedomorphs have a similar pattern of limb integration. The correlation coefficients between morphs for forelimb elements ($r = 0.95$ for raw and $r = 0.99$ for scaled data), hindlimb elements ($r = 0.94$ for raw and $r = 0.99$ for scaled data) and for both fore- and hindlimb elements ($r = 0.63$ for raw and $r = 0.98$ for scaled data) were high and significant for almost all comparisons (see Table 3 for details within each morph).

Partial correlation analysis - Within limbs

Comparison of partial correlation matrices between morphs for all limb elements showed high similarity: the correlation coefficients between morphs for all limb elements (both fore- and hindlimb elements) ($r = 0.72$, $P < 0.05$), forelimb elements ($r = 0.82$, $P > 0.05$), hindlimb elements ($r = 0.86$, $P < 0.05$). There were few consistent patterns of partial correlations in both morphs. For the forelimbs, significant partial correlations (edges) were present between humerus and radius and metacarpal and first phalanx. Correlation strength between humerus and radius was higher in paedomorphs, whereas in metamorphs it was higher between metacarpal and first phalanx (Fig. 2, Table 4). In hindlimbs, significant edges between femur and tibia and metatarsal and first phalanx were common for both morphs with higher strength in paedomorphs (see Fig. 2, Table 4).

Table 3. Correlation matrices and results of matrix correlation analysis for fore- and hindlimbs of metamorphs and paedomorphs in *I. alpestris*. A) Correlation matrix for metamorphs (left to the diagonal) and paedomorphs (right to the diagonal); B) Results of matrix correlation analysis (left to the diagonal) and Mantel's test (*p* values in italics right to the diagonal). Results for scaled data are given in parenthesis. See Table 2 for the abbreviations of bones.

A)

	H	R	MC	Ph 3.1	F	T	MT	hPh 3.1
H	1	0.92	0.75	0.69	0.68	0.64	0.6	0.55
R	0.85	1	0.74	0.66	0.63	0.67	0.62	0.54
MC	0.58	0.53	1	0.82	0.48	0.49	0.71	0.68
Ph 3.1	0.37	0.36	0.83	1	0.49	0.5	0.65	0.66
F	0.88	0.77	0.51	0.36	1	0.86	0.68	0.55
T	0.74	0.66	0.55	0.38	0.79	1	0.71	0.58
MT	0.62	0.62	0.66	0.54	0.67	0.73	1	0.89
hPh 3.1	0.55	0.52	0.69	0.56	0.6	0.69	0.85	1

B)

	Metamorph		Paedomorph	
	forelimb	hindlimb	forelimb	hindlimb
Metamorph forelimb		0.12 (0.00)	0.00 (0.04)	0.04 (0.00)
Metamorph hindlimb	0.87(0.96)		0.11 (0.05)	0.00 (0.00)
Paedomorph forelimb	0.95 (0.99)	0.74 (0.96)		0.04 (0.04)
Paedomorph hindlimb	0.98 (0.97)	0.94 (0.99)	0.90 (0.98)	

Partial correlation analysis - Between limbs

In both morphs significant partial correlations (edges) was found between humerus and femur. In paedomorphs, additional significant edges appeared between radius and femur, radius and tibia, metacarpal and metatarsal. Focusing on partial correlations among serial homologues, the humerus and femur showed significant correlations in both morphs with higher strength in metamorphic individuals. No other significant edges were observed between serial homologues in metamorphic individuals, whereas in paedomorphic individuals significant edges were found for all serial homologues, except for most distal elements/phalangeal elements.

Table 4. Partial correlations (left to the diagonal) with corresponding edge exclusion deviance (EED) values (right to the diagonal) in *I. alpestris*. Numbers in bold indicate significant results corresponding to $p = 0.05$, d.f. = 1, from the χ^2 -distribution. See Table 2 for the abbreviations of bones.

A)

Metamorphs	H	R	MC	Ph 3.1	F	T	MT	hPh 3.1
H		59.79	2.5	0.63	15.18	3.43	2.79	0.92
R	0.77		1.24	0.04	6.23	9.04	1.11	1.37
MC	0.19	0.14		21.4	1.42	0.99	5.62	0.01
Ph 3.1	0.1	-0.02	0.53		0.03	0.13	0.72	2.79
F	0.45	-0.3	-0.15	0.02		41.03	3.48	1.05
T	-0.22	0.36	-0.12	0.04	0.68		2.25	0.01
MT	-0.2	0.13	0.29	-0.1	0.23	0.18		58.49
hPh 3.1	0.12	-0.14	-0.01	0.2	-0.13	-0.01	0.77	

B)

Paedomorphs	H	R	MC	Ph 3.1	F	T	MT	hPh 3.1
H		19	5.9	1.73	24.85	0.89	0.84	0.36
R	0.54		0.08	0.08	0	0.05	2.14	0.19
MC	0.32	-0.04		44.06	2.23	0.09	0.18	2.79
Ph 3.1	-0.18	0.04	0.75		0.49	0.35	0.14	0.01
F	0.61	0	-0.2	0.09		4.79	1.15	0.12
T	0.13	-0.03	0.04	-0.08	0.29		2.02	1.6
MT	-0.12	0.2	0.06	0.05	0.15	0.19		25.11
hPh 3.1	-0.08	-0.06	0.22	-0.01	0.05	0.17	0.61	

Average partial correlations showed higher associations within each limb than between limbs in both morphs, but with greater decline for between limb partial correlations in metamorphs (Table 5; Fig. 2). The decline in the level of between limb correlation in metamorphs can be seen more clearly by looking at the average correlation of homologous limb elements where ratio of within and between limbs was in metamorphs 1.563 and in paedomorphs near one (0.960). The ratio between average partial correlations for forelimbs to hindlimbs revealed further differences between morphs. In metamorphic individuals this ratio exceeded unity indicating higher correlations of forelimb to hindlimb elements. A different pattern was found in paedomorphs in which much higher correlations occurred within hindlimb elements than within forelimbs (Table 5).

Table 5. Average partial correlations within and between limb skeletal elements in alpine newt metamorphs and paedomorphs. The ratio of partial correlations within and between limbs, within and between homologous limb elements and between fore- and hindlimb is also presented.

	Metamorphs	Paedomorphs
Within forelimb	0.311	0.291
Within hindlimb	0.243	0.333
Forelimb/hindlimb ratio	1.281	0.876
Within limbs	0.277	0.312
Between limbs	0.125	0.179
Between homologous limb elements	0.177	0.325
Within/Between ratio	2.222	1.744
Within/Between homologous limb elements ratio	1.563	0.96

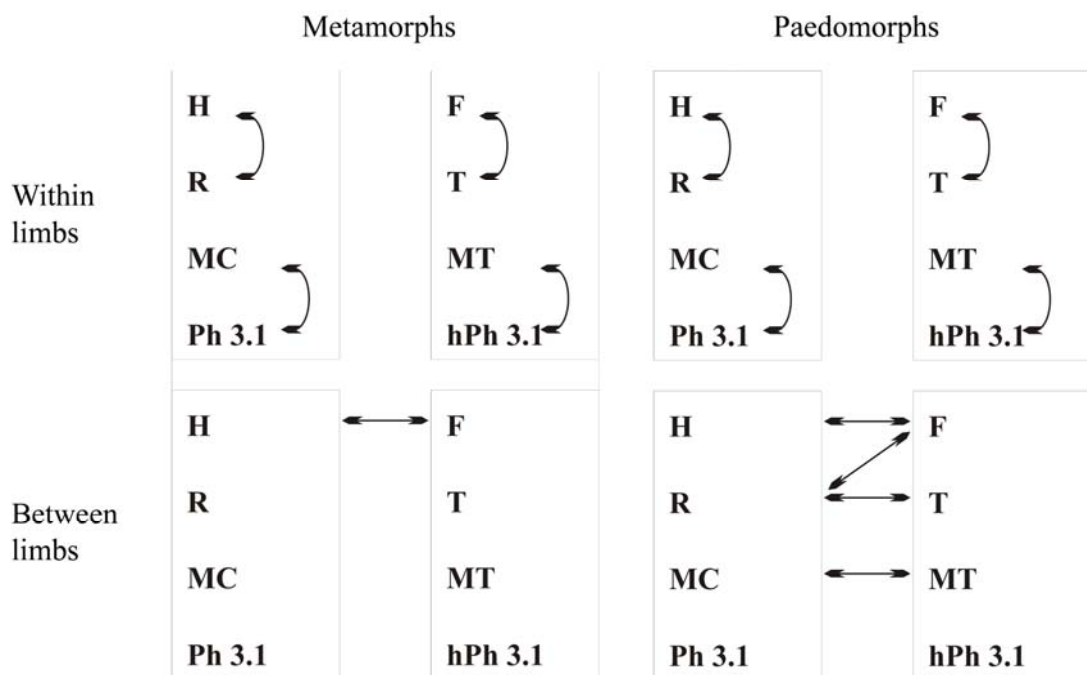


Figure 2. Schematic diagram of fore- and hindlimbs from metamorphs and paedomorphs showing the significant partial correlations between elements ($P < 0.05$). The arrows point to associations between traits within and between fore- and hindlimbs. Forelimb: H - humerus, R - radius, MC - metacarpal of the third digit, Ph 3.1 - first phalanx of the third digit; Hindlimb: F - femur, T - tibia, MT - metatarsal of the third digit, hPh 3.1 - hindlimb first phalanx of the third digit.

Variance of the eigenvalues (VE) was used to assess overall covariation of limb elements. The index of integration for metamorphs was 0.471 (0.383 for scaled data) and for paedomorphs 0.511 (0.379 for scaled data), with a statistically significant difference between morphs considering raw data ($P = 0.022$), but not for scaled data ($P = 0.826$).

Evolvability

The mean cosine of the angle between the selection and response vector of the limbs was for metamorphs 0.500 (0.512 for scaled data) and for paedomorphs 0.502 (0.533 for scaled data). Statistically significant difference in evolvability was found only for scaled data (raw data: $P = 0.552$; scaled data: $P = 0.002$).

DISCUSSION

Patterns of integration

The patterns of limb correlation in paedomorphs and metamorphs of the alpine newt indicate that they share the same general pattern of morphological integration. Major differences between the morphs were found for the partial correlation of fore- to hindlimb elements and the patterns of partial correlation between the fore- and hindlimbs. The paedomorphs have a greater number of correlations between limbs, especially those between the homologous limb elements. Although paedomorphs have a higher index of overall integration, when size was removed the difference between morphs was negligible. This indicates a large impact of size-related variation on integration patterns.

In general, high matrix correlations between limbs would indicate that developmental integration has an important impact on limb integration (e.g. Young and Hallgrímsson, 2005; Schmidt and Fischer, 2009). Therefore, such a high integration between homologous limb elements of fore- and hindlimbs in paedomorphs could be explained by dominance of developmental patterning. Evolutionary studies of morphological integration showed that different functional demands on fore- and hindlimbs can influence developmental and genetic constraints, reducing between limb integration and leading to independent specialization of fore- and hindlimbs in various groups (Hallgrímsson et al., 2009; Young et al., 2010; Kelly and Sears, 2011; Bell et al., 2011). In mammals, disparity in patterns of correlations between fore- and hindlimbs were also explained by different developmental timing of fore and hindlimbs (Bininda-Emonds et al., 2007). Compared with mammals and birds, limbs in newts and salamanders have largely similar functions, although hindlimbs have the major, propulsive role in terrestrial walking. Moreover in such species, there is dissociation in the timing of limb development, as development of the forelimbs is accelerated relative to the hindlimbs. This disparity is very pronounced, as

forelimb chondrification is finished before the hindlimb bud has even appeared (Glücksohn, '32; Knight, '38; Bininda-Emonds et al., 2007). However, these factors cannot be directly related to the observed differences in the pattern of integration and dissociation of fore- and hindlimbs in metamorphosed adults. As in populations of facultatively paedomorphic newts, both syntopic morphs have a similar genetic background and early developmental pathways (see also Oromi et al., 2016). However, these factors could indirectly alter correlation patterns through changes in growth patterns under the influence of different mechanical loading during terrestrial locomotion and aquatic locomotion. Indeed, the variation in bone growth is directly related to local parameters of mechanical stress and strain (Hall, 2005 and references therein). Bone adapts to a pattern of daily mechanical loading and is sensitive to changes in this normal pattern, altering mass, and morphology in response to the new forces (Frost, '88; Raab-Cullen et al., '94). However, the results of the empirical study of Young et al. (2009) indicates that factors such as neuromuscular coordination or bone-muscle interactions do not play a role in the structure or magnitude of limb integration.

Aquatic versus terrestrial life constraints

Differences in correlation patterns between both morphs could be related to their different locomotion mode. The aquatic mode of locomotion in paedomorphs (as in all aquatic salamanders during swimming) is primarily achieved by anguilliform locomotion and propulsive movements of the tail, with no participation from the limbs during swimming (Frolich and Biewener, '92; Ashley-Ross and Bechtel, 2004). However, the existence of aquatic walking could also affect the correlation structure. As shown by Deban and Schilling (2009), terrestrial and aquatic walking have similar temporal patterns of muscle activation. This suggests that the basic functions of the trunk muscles in walking are conserved across environments. However, muscle recruitment is greater during terrestrial walking due to greater gravitational forces, making terrestrial walking (where limbs must support the weight of the body and allow movement in several directions) more demanding relative to aquatic walking (Deban and Schilling, 2009). An empirical study of locomotion in paedomorphic *Ambystoma tigrinum* (Frolich and Biewener, '92) found similarities during swimming in both morphs (similar axial wave patterns despite significant differences in axial morphology), but metamorphosed adult individuals employed two distinct axial motor programs, one for swimming and the other one for walking. Different motor programs and forces produced by muscles attached to the bones could affect and direct bone growth (Frolich and Biewener, '92; Deban and Schilling, 2009; Zelditch et al., 2004; Hallgrímsson et al., 2009; Klingenberg, 2014). In metamorphs, limbs provide forward propulsion while the autopod as distal limb element is in contact with the substrate. This is particularly true for hindlimbs that generate propulsive force and may be strongly influenced by functions related to locomotion and consequently may respond most strongly to loads. The absence of significant partial correlations between more distal fore- and hindlimb elements in metamorphs may reflect this biomechanical effect.

Bone remodeling due to increased muscle loadings and the body support necessary in terrestrial locomotion is well documented (Biewener, '90; Blob and Biewener, '99; Sheffield and Blob, 2011). Bone tissue replacement and bone remodeling are very common in salamanders (Lebedkina, 2004). Therefore, the complexity of terrestrial locomotion and the existence of a return to the aquatic environment accompanied with aquatic locomotion in metamorphic individuals is a powerful factor that may affect the correlation structure and lead to dissociation of fore- and hindlimbs. The increased calcification related to metamorphosis (Smirnov and Vassilieva, 2003) followed with increased muscle loadings necessary for body support in terrestrial locomotion, could affect the correlation pattern.

Evolvability

Evolvability captures the ability of a population mean to respond to a selective challenge (Rolian, 2009). The results based on raw data shows that limbs of both morphs were similarly evolvable. As was noted by Rolian (2009), size-related variation significantly impacts evolvability. Based on scaled data, limb evolvability increased in both morphs once the effect of size was removed, with stronger effect for metamorphs. This means that body-size related variation acts as a form of constraint, channeling phenotypic responses along the axis of least resistance in the phenotypic space (Schluter, '96; Marroig and Cheverud, 2005; Rolian, 2009). Once size-related variation was removed, the limb means could respond more freely in the direction of selection. Higher levels of evolvability in metamorphs were not accompanied with lower overall integration as was shown in different studies, mainly mammalian species (Rolian, 2009; Lewton, 2012). But, lower between limb correlations in metamorphs can be regarded as dissociation (achieved through a reduction in pleiotropic interactions among these variables) which further could lead to more evolvable phenotype.

Our present work shows that facultative paedomorphosis is linked to changes in the correlation structure through different aquatic vs. terrestrial constraints that shape the patterns of morphological integration and evolvability. Because of the potential changes of integration across specific life stages, future studies should determine integration patterns at all relevant life stages across ontogeny. Such studies would also help us to understand differences in developmental trajectories of paedomorphic and metamorphic newts and salamanders, and to decipher the influence of developmental and functional constraints on the pattern of morphological integration in newt and salamander limbs.

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