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Can heterochrony help explain the high morphological diversity within the genus *Niphargus* (Crustacea: Amphipoda)?

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Abstract

Postembryonic development of nine species of *Niphargus* (Crustacea: Amphipoda) was studied, with spine development and shifts in allometric growth being combined in developmental sequences that were compared across species. The developmental sequences show high diversity with respect to the position of individual events in the sequence, as well as a high frequency of events being inapplicable in some species. Within *Niphargus*, the highest degree of independence between events occurs mainly in early mid-aged instars, where the shifts in position are both the largest in magnitude and also the most frequent in occurrence. Constructive troglomorphic features of subterranean species were inferred to develop more because of accelerated growth rather than a delayed offset of growth. Shifts in both relative timing and growth rate appeared to have played a role in the evolution of sexually dimorphic elongated appendages. Growth patterns differed greatly between species for individual sexually dimorphic and troglomorphic traits, hinting at their possible independent origin. The independence between developmental events that was generally apparent might indicate the existence of an important genetic basis for the extreme intrageneric morphological variation arising from numerous highly variable body parts being combined in a 'mosaic' manner. Moreover, we suggest that the overall diversity of the genus might actually be underestimated in light of possible convergent features accompanied by 'cryptic' speciation. We conclude that both sequential and growth heterochrony appear to have played a key role in the evolution of *Niphargus*, the most diverse genus of freshwater amphipods. A preliminary list of heterochronic characters is provided.

Keywords: *Niphargus*; Heterochrony; Postembryonic development; 'Cryptic' speciation; Phenotypic diversity

Introduction

Changes in developmental timing (heterochrony) have been held to play an important, if disputed, explanatory role in evolutionary biology since Haeckel (Gould 1977). Originally, they were considered as a discrepancy that disrupted the recapitulation of phylogeny in ontogenetic development (citations in Gould 1977; Richardson and Keuck 2002). Thereafter, de Beer attempted to refute the concept of recapitulation and demonstrated how changes in the relative timing of events or rates of

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processes could generate differences among organisms (reviews in Gould 1977; Smith 2001, 2002, 2003). Much of the modern concept of heterochrony derives from Gould (1977) who, unlike de Beer, returned to linking recapitulation and heterochrony, with the latter representing a mechanism that produces a parallel between ontogeny and phylogeny. His view on heterochrony was based on a dissociation of growth, differentiation and maturation. However, his 'clock-analysis' had a limited analytical framework. Alberch et al. (1979) later refined Gould's concept, improved the analytical framework and extended the terminology. Together, the two works, which emphasized the modification of forms or shapes ('allometric heterochrony'), founded the context in which heterochrony is still largely viewed today, with the concept being used mainly as an explanatory tool for the evolution of individual characters within well-established ancestor–descendant relationships. The analytical methods developed, however, allowed neither assessment of the phylogenetic relationships among taxa nor the simultaneous study of several characters. The sole early attempt to use heterochrony in cladistic analyses as a criterion for the polarization of characters (Nelson 1978) encountered severe criticism (Alberch 1985; Kluge 1985).

More recently, increased interest among developmental biologists for incorporating an evolutionary framework in their research revived de Beer's and Haeckel's approaches toward heterochrony that emphasized the differential timing of developmental events ('sequence heterochrony'). In the late 1990s, several authors independently proposed use of the relative timing of events as a method to encode heterochrony data such that the latter could be interpreted with the help of known phylogenies (Mabee and Trendler 1996; Smith 1997; Velhagen 1997; see also Jeffery et al. 2002a, b, 2005), or analyzed statistically (Nunn and Smith 1998) or with help of so-called dissociation plots (Schlosser 2001) (reviews of all methods in Richardson et al. 2001; Bininda-Emonds et al. 2002). In addition, several studies confirmed that developmental sequences apparently contain at least some phylogenetic signal (Velhagen 1997; Koenemann and Schram 2002; Jeffery et al. 2002b; Smirthwaite et al. 2007), although the amount of signal appears to vary among groups (see Bininda-Emonds et al. 2003a) and the ability of current methods to reliably extract any signal has been called into question (Schulmeister and Wheeler 2004).

To date, the majority of methodological and empirical research into sequence heterochrony has focused on events in the early embryogenesis of different vertebrate groups (e.g. most of the studies cited in the previous paragraph). In the present study, we applied the same methods to analyze postembryonal development in nine species of the predominantly subterranean amphipod genus *Niphargus* Schiödte, 1847. With over 300 described species and subspecies, this genus is the largest among all freshwater amphipods (Pinkster 1978; Karaman and Ruffo 1986; Fišer et al. 2002; Väinölä et al. 2008). Most of the known species inhabit subterranean waters and constitute a substantial part of the European groundwater biodiversity (Sket 1999a, b). The genus is distributed across most of Europe, mainly but not exclusively south of the Pleistocene ice sheet boundary (Ruffo 1953a; Karaman and Ruffo 1986; Proudlove et al. 2003). Few species are known from the Arabian Peninsula, Turkey and Iran (Karaman 1986, 1998, 2003; Bat et al. 2000), whereas only its presumed relative, *Haploginglymus* Mateus & Mateus, 1958, can be found over most of the Pyrenean Peninsula (Karaman and Ruffo 1986).

Niphargus displays extremely diverse morphology (Karaman and Ruffo 1986; Sket 1999a) that can be attributed largely to numerous highly variable body parts being combined in a high number of combinations (hereafter called a 'mosaic'; see e.g. Sket and Notenboom 1993). This diversity often occurs in a highly confusing pattern. For instance, differences between presumably closely related species are often comparatively small and can be overlooked easily (Fišer in preparation), whereas the variability of more distinctive characters within a species is frequently high. Furthermore, those characters that are hypothesized to be adaptive to subterranean environments (troglomorphic characters or troglomorphies) are believed by many to often arise convergently as a consequence of the homogenous selection forces acting in such environments (Langecker 2000; Aden 2005; Christiansen 2005). Finally, certain appendages in several species appear in late ontogeny in a remarkably modified form (Ginet 1960a, b; Morand 1974), such that the juvenile specimens of these species can appear more similar to the adults of completely unrelated species than to adults of their own species (e.g. Jersche 1963; Sket 1974).

In this paper, we first provide an overview of some characters that have possibly undergone an evolutionary change as a result of heterochrony. Rather than discussing each character within the context of Gould (1977) or Alberch et al. (1979), we translated the morphological changes occurring in late ontogeny into a series of discrete events that could be analyzed as a developmental sequence. In doing so, we sought to address the basic question of how independent of the rest of events across the species the timing of any single event is. A key methodological issue here was the problem how to reduce an allometric curve to a single event that could be embedded in a developmental sequence. The resulting developmental sequences were very heterogeneous, which allowed us address the importance of sequence heterochrony for understanding the high morphological variability within *Niphargus*. Finally, the comparison of onset, offset and growth rates in the development of selected characters provided new revelations with respect to troglomorphic characters, sexual dimorphism and apparent 'cryptic' speciation where we are unable to recognize suitable morphological features distinguishing species.

Material and methods

Taxonomic sampling

As part of another study involving revision of the genus (Fišer et al. in preparation), it became clear that several characters might have evolved as a result of heterochronic changes. From these 60 taxa (not listed here) we compiled a preliminary list of potentially heterochronic characters (see the following text section) and sought to study a subset of these characters in greater detail on selected species only. For the latter, taxonomic sampling was designed to include both taxa among which developmental patterns differ only slightly and taxa among which remarkable differences in late ontogeny patterns exist. We were able to obtain complete or partially complete developmental sequences for nine taxa that fulfilled these goals: *Niphargus costozzae* Schellenberg, 1935; *N. hebereri* Schellenberg, 1933; *N. krameri* Schellenberg, 1935; *N. longicaudatus* Costa, 1851 from Cres Island (sensu Karaman 1989); *N. longicaudatus* from Napoli; *N. longiflagellum* S. Karaman, 1950; *N. salonitanus* S. Karaman, 1950; *N. spinulifemur* S. Karaman, 1953; and *N. spoeckeri* Schellenberg, 1933. Straškraba (1972) assigned these nine taxa to four species groups: *stygius-puteanus*, *skopljensis*, *longicaudatus* and *orcinus* (Table 1). Thus, taxonomic sampling across *Niphargus* is highly incomplete, largely because juvenile specimens could not be obtained for several other major morphotypes such as the species groups *aquilex*, *carpathicus*, *jovanovici* and *kochianus* sensu Straškraba (1972).

All taxa examined have the taxonomic rank of species, with the exception of the two *N. longicaudatus* populations. *Niphargus longicaudatus* has one of the largest distribution ranges among niphargids (Barnard and Barnard 1983), comprising Corsica (as *N. l. corsicanus*), northern Italy (as *N. l. thuringius* and *N. l. debilis*), central-southern Italy and the eastern Adriatic coast (as the nominotypical subspecies), and Greece (as *N. l. versluysi*). *Niphargus longicaudatus* s.str. has the largest range among the five subspecies – a very unlikely feature for a subterranean species (e.g. Sket et al. 2004). However, 'cryptic' speciation, similar to that demonstrated recently for *N. virei* (Lefébure et al. 2006), can also be revealed for *N. l. longicaudatus* (Trontelj et al. 2007). The Italian and Croatian populations resemble one another closely (Karaman 1989; personal observation by C.F.), so that they appear to make an appropriate model for assessing the identity of taxa on the basis of developmental patterns.

The numbers of specimens examined for each taxon together with their collection locations are listed in Table 1. Where possible, specimens of all sizes between

Table 1. Overview of *Niphargus* specimens examined

Taxon	*N*	Species group (Straškraba 1972)	Locality
N. costozzae Schellenberg, 1935	24	*stygius-puteanus*	ITA, Berici Mt., Cave Grotta della Guerra; 22.9.2002; leg. P. Trontelj, B. Sket, C. Fišer
N. hebereri Schellenberg, 1933	28	*skopljensis*	CRO, vicinity of town of Rovinj; one sample each from 1977, 1979, 1980
N. krameri Schellenberg, 1935	44	*stygius-puteanus*	CRO, vicinity of town of Pazin; three samples from 2002–2003; leg. C. Fišer
N. longicaudatus Costa, 1851[a]	25	*longicaudatus*	ITA, Napoli, spring near road Monte Faito to Vico Equense; 26.3.2004; leg. C. Fišer
N. longicaudatus-Cres	28	*longicaudatus*	CRO, Cres Island, Lubenice village, Retec spring; 30.4.04; leg. B. Sket
N. longiflagellum S. Karaman, 1950	25	*orcinus*	SLO, Dobrepolj, Podpeška jama cave; 1970, 1971, 1985
N. salonitanus S. Karaman, 1950	16	*orcinus*	CRO, Split, Kaštel Stari, spring near Stomarija church; 27.4.2004; leg. Kralj & Fišer
N. spinulifemur S. Karaman, 1953	48	*stygius-puteanus*	SLO, vicinity of border pass of Sočerga; four samples from 2002–2003; leg. C. Fišer
N. spoeckeri Schellenberg, 1933	23	*stygius-puteanus*	SLO, Postojna, Pivka jama cave; 4.3.1993; leg. F. Velkovrh

The taxonomy used follows current opinion and should not be taken to imply that all groups are accurately defined and natural. *N* = number of specimens.

[a] Referred to as *N. longicaudatus*-Napoli in the text.

marsupial juveniles and the largest adults were examined (see also under Section "Temporal component"), although the actual number and sizes of specimens studied obviously depended on the availability of material. For some rare species, samples from several geographically close localities were pooled.

All specimens used in this study have been deposited in the collection of the Oddelek za biologijo, Biotehniška fakulteta, Univerza v Ljubljani (Department of Biology, Biotechnical Faculty, University of Ljubljana, Slovenia). They were macerated in a 10% KOH solution at 100 °C, rinsed briefly with diluted HCl, and washed in distilled water. Cleared exoskeletons were stained with chlorazol black in glycerol, partly dissected in glycerol, and mounted on slides in a glycerol–gelatine medium. Digital photographs were taken with an Olympus DP10 camera mounted to an Olympus SZX9 stereomicroscope. Measurements and counts were made using the computer program Olympus DP-soft. Finer details were examined using a Zeiss microscope at magnifications of 100–400x.

Preliminary list of characters possibly influenced by heterochrony

In compiling the initial list (see Appendix), we included those features for which the character states can be found in both adult specimens of some species (designated as a, a′, a″, …) and as part of the transformational series in the postembryonic development of other species (designated as a → a′ → a″, …). However, in so doing we do not claim a priori that the similarities between the juveniles and adults of the different species are a consequence of heterochronic processes (see Webster and Zelditch 2005).

The list is arranged as a generalized description, with characters derived from the body and appendages described in turn. For each character, we provide a case species in which the postembryonic transformation series was traced and, where possible, also cases in which the character appears to be paedo- or peramorphic. Note that we refer to these latter expressions literally (i.e. as juvenile-like and hyper-developed, respectively), not as synonyms for the respective terms plesiomorphic and apomorphic. We do not address the direction of evolutionary transformation in the characters (e.g. acceleration versus retardation), which cannot be established until a reasonable phylogeny (if not taxonomy!) within the genus is established. Unfortunately, the phylogenetic affinities of, and relationships within, *Niphargus* remain largely unknown despite being discussed by a number of authors (e.g. Sket 1981). It seems likely, however, that substantial taxonomic revision will be necessary in the future, given the demonstrated paraphyly of the *orcinus*-group (Fišer et al. 2006a) and also that several ongoing studies indicate that the monophyly of many other proclaimed subgeneric groups is even more questionable.

The list of characters we present is, to our knowledge, one of the first efforts in this direction for this group of organisms. As such, many uncertainties and doubts about these characters undoubtedly exist, but their study is beyond the scope of the present paper. Instead, we present the list in Appendix as a simple working sheet, with the hope that it will encourage other researchers in the field to further study.

Events

We refer to an 'event' as a change in the morphology of a character (see Appendix) that occurs during development (e.g. a shift in growth rate or the development of a particular spine). As such, it is possible for several events to apply to the same, single character. An 'inapplicable event' is one that does not occur in the focal species, because either the event itself or the character it applies to is absent. It is thus distinguished from the case in which the event can minimally be inferred to occur, but could not be characterized precisely because the necessary specimen was not sampled ('missing data'). We studied 20 events, which are of three types.

Type I events are the simplest and are characterized by the development of selected setae or spines. For some spines, the development of the first spine/setal group occurs in one of the earliest instars that were absent from most of our samples. In such cases, the event used in the analyses refers to the development of the second spine rather than the first.

Events of type II describe changes in size and shape as quantified with an index derived from allometric plots of appendage length versus body size (Fig. 1). All non-linear growth curves were described with a model using two regression lines, placed empirically such that the residual variance of the bilinear model (M_c) was minimized compared to a single-line model (the null model, M_0). As an initial starting point, the first regression line described the growth of the three smallest specimens, with the second line summarizing that of the remaining, larger animals. Thereafter, specimens from the second group were transferred to the first in stages, and the boundary between the two groups was successively increased by an interval of 1 mm until the second regression line was constructed from the (last) largest three specimens. The proportion of unexplained (residual) variance between bilinear and null models is given by $V_c = \sigma_c^2/\sigma_0^2$, where σ_c^2 and σ_0^2 express the residual variances of bilinear and null models, respectively, which we used to determine whether the bilinear model provided a statistically better fit to the data,

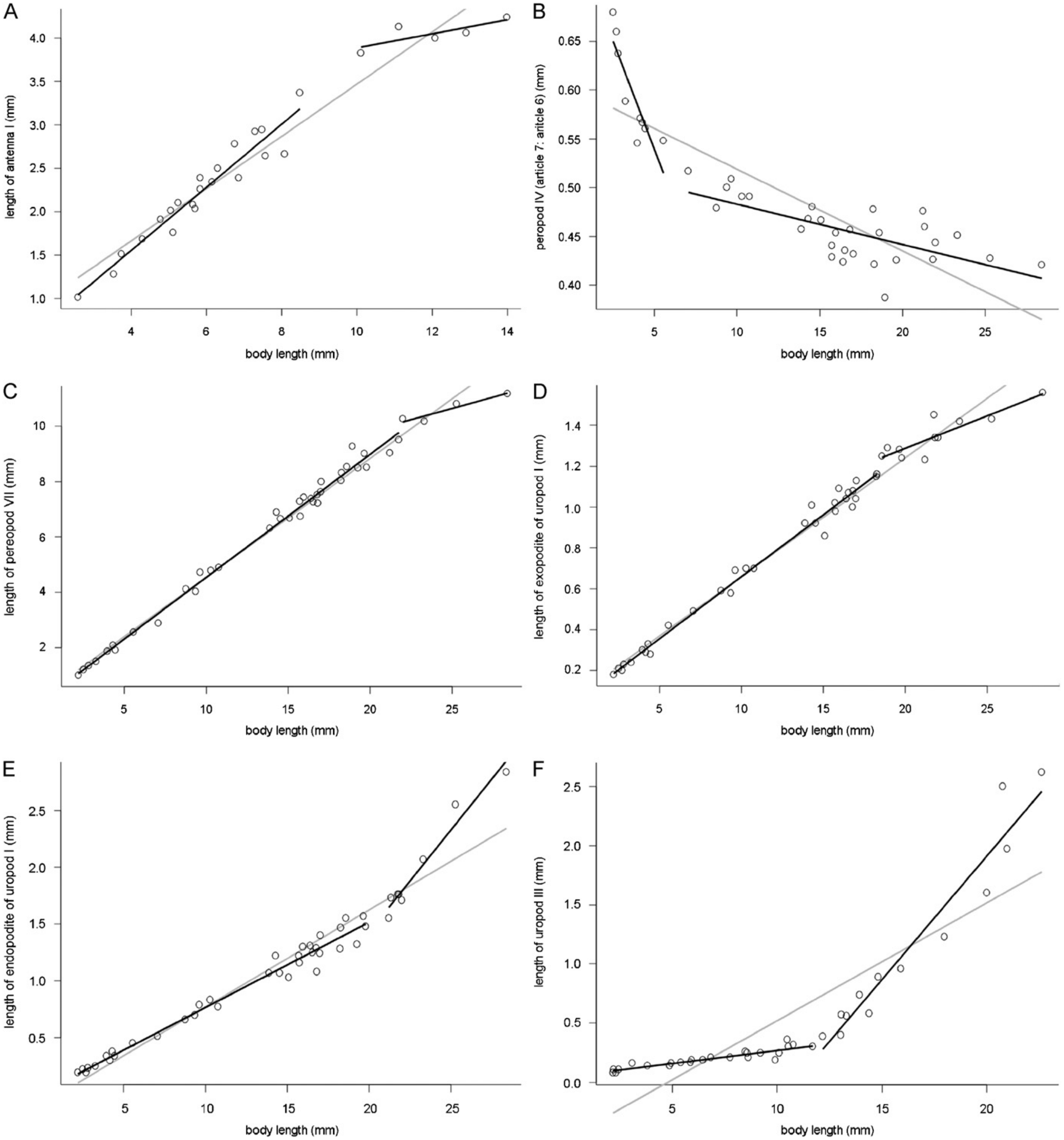

Fig. 1. Exemplar growth curves, best-fitting regression lines and overall regression lines. (A) Antenna I (*N. longicaudatus* from Cres). (B) Dactylar index of pereopod IV (art. 7/art. 6) (*N. krameri*). (C) Pereopod VII (*N. krameri*). (D) Exopodite of uropod I (*N. krameri*). (E) Endopodite of uropod I (*N. krameri*). (F) Distal article of exopodite of uropod III (*N. spinulifemur*). Gray lines: single-line models; black lines: bilinear-regression model.

taking into account the different number of parameters each model uses on the basis of the Akaike Information Cirterion (AIC).

The intercept of the two lines of the best fitting bilinear model, which we refer to as the ‘critical point’, estimates the imaginary size (or age; see below) at which differentiation or a change in the growth trajectory takes place in the species in question. We further assumed that some significant change in the growth process of the character occurs at this point, thereby defining a heterochronic ‘event’. If the line is linear (such that a critical point could not be identified), the relevant event was considered to be inapplicable.

Events of type III describe changes in the shape of the propodi (articles 6) of gnathopods I and II. The highly variable shape of the propodus was modeled as a triangle (see Fig. 2) defined by the propodus length (= distance between the carpus–propodus and propodus–dactylus joints, as measured on the outer side; Fig. 2, *l*), the palm length (= distance between the dactylus–propodus joint and the base of the palmar spine; Fig. 2, *p*), and the diagonal length (= distance between the carpus–propodus joint and the base of the palmar spine; Fig. 2, *d*). Differentiation in the shape of the propodus can result from unequal growth rates in any or all three parameters, with the shape of the ‘triangle’ changing over both ontogenetic and phylogenetic timescales. Given that the ontogenetic changes in this character appear to be gradual, we defined two events based on the following length ratios involving all pairwise combinations of the three parameters: (a) palm/propodus length, (b) diagonal/propodus length and (c) diagonal/palm. The complete developmental sequence for this character typically proceeds as $a<b<c \rightarrow a>b<c$ (the relationship between a and c is not fixed; a is gradually getting larger than c) $\rightarrow a>b>c$ (shown graphically in Fig. 2), with the relative timing of each the two transformations serving as individual events (referred to in Table 2 as transformations 1 and 2). Note, however, that the terminal stage(s) is(are) not necessarily present. In other words, one or both of the transformations might be absent such that the associated events would be scored as being inapplicable. This representation of the shape of the propodus is admittedly somewhat rudimentary, but served as a useful exploratory tool that will hopefully give additional insight into the precise growth patterns underlying the transformations (see also Discussion).

Table 2. Developmental events examined

Label	Event
A[a]	Development of second spine on urosomite I
B	Development of second spine on urosomite II
C	Development of second spine on epimeron II
D	Development of second spine on epimeron III
E[a]	Development of first additional group of setae anteriorly on carpus of gnathopod I
F	Development of first additional group anteriorly on carpus of gnathopod II
G	Development of first spine in telson cleft
H	Development of first lateral spine on telson
I	Development of first dorsal spine on telson
J[a]	Development of fourth apical spine on telson
K	Offset of growth (length) of antenna I (see Fig. 1A)
L	Transformation 1[b] of propodus shape of gnathopod I
M[a]	Transformation 2[b] of propodus shape of gnathopod I
N	Transformation 1[b] of propodus shape of gnathopod II (see Fig. 2)
O[a]	Transformation 2[b] of propodus shape of gnathopod II (see Fig. 2)
P	Shortening of dactylus of pereopod IV (length ratio art. VII/VI) (see Fig. 1B)
Q	Offset of growth (length) of pereopod VII (see Fig. 1C)
R[a]	Onset of elongation of endopodite of uropod I (see Fig. 1E)
S[a]	Offset of growth (length) of exopodite of uropod I (see Fig. 1D)
T	Onset of elongation of exopodite of uropod III (see Fig. 1F)

For a review of appendicular morphology and its interspecific variability, see Karaman (1993).

[a] Event inapplicable in three or more species.

[b] Transformations similar for both pairs of gnathopods (see text and Fig. 2).

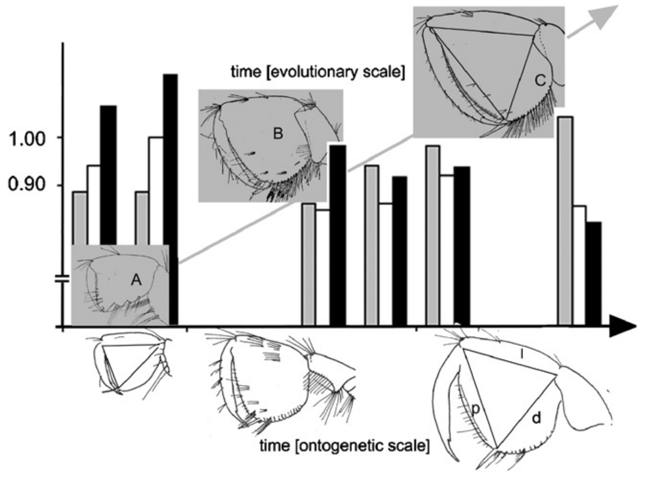

Fig. 2. Concordance between ontogenetic and evolutionary change in transformation of shape of gnathopod II as measured by three length ratios: gray bars = palm (length *p* at bottom right)/propodus (length *l*); white bars = diagonal (length *d*)/*l*; black bars = *d*/*p*. Ontogenetic scale presented on *x*-axis, using six ontogenetic stages in *N. costozzae*. Transformation 1 occurs between stages 1–2 and 3–5 in this species, transformation 2 between stages 3–5 and stage 6. Same transformation series also describes difference between three species on an evolutionary scale: A = *N. tamaninii* (after Ruffo 1953b); B = *N. maximus* (after Karaman 1943) and C = *N. orcinus* (after Karaman 1950).

Temporal component

Given that niphargids, like all arthropods, grow and differentiate in discrete molting cycles, the most convenient method for the temporal alignment of events in this group arguably would be the analysis of successive instars. Doing so obviates many of the problems identified by Bininda-Emonds et al. (2002) with the definition and use of developmental stages, but still makes the strong assumption that a given instar stage is homologous across species. Even so, other researchers have found the direct comparison of instars to still be problematic in practice (Minelli and Fusco 1995). Moreover, the current study was conducted on preserved samples, in which the identification of instars is extremely difficult and possible only when abundant samples are available (500–600 specimens; see Chapelle 1995; Kokkotis and McLaughlin 2002). Instead, size seems to be a reasonable estimator of age for events of type II (bearing in mind the caveats of Bininda-Emonds et al. 2002), because the regression lines summarize differentiation over the entire sample and do not depend on the exact placement of any particular individual within its instar. The temporal sequence of events of types I and III was identified using the "consensus method" of Velhagen (1997), which indicated again that size provides a reasonable estimate of the presumed age of a specimen for these data.

Again, the lack of a robust phylogeny for *Niphargus* meant that the comparison of developmental sequences between species could not be performed in a phylogenetic framework. Thus, we used rank order to study temporal shifts on a relative scale, and size to approximate absolute timing standardized in an interval between 0 and 1. For the latter, the largest specimen was considered to be the oldest in the sample (time = 1) and the duration of 'temporal subperiods' (e.g. early half and late half; see following section) defined the size classes (e.g. specimens smaller or larger than half of the largest, respectively). Because the delineation of all size classes relies crucially on the size of the largest specimens, the reliability of our data for the latter was verified with data from the literature.

Comparison of developmental sequences

The aim of this analysis was to investigate the extent to which events were dissociated from one another, to identify the period in which dissociation is the highest, and to investigate the frequency and distribution of inapplicable events. For these purposes, we followed methodological premises of Nunn and Smith (1998) and Bininda-Emonds et al. (2003b).

To study the distribution of inapplicable events, we ranked events according to their position in the developmental sequence (see Nunn and Smith 1998). Events occurring in a sequence simultaneously were given the average rank of the set of simultaneous events. Truly inapplicable events were left unranked, whereas allowances needed to be made for events that must have occurred in a missing part of a developmental sequence. For example, we had several marsupial specimens for *N. costozzae* (undifferentiated), but lacked juvenile specimens from the size class between 3 and 7 mm representing the period during which events ABCD obviously occur (see Table 3). Thus, new ranks were inserted for this missing size class (one for each apparently missing event), and each missing event that must have occurred in this interval was given the average of the inserted ranks. Ranked events across the species are not shown, but can be obtained from the authors on request (they can also be extracted from Table 3). Subsequently, the average rank of any given event was calculated across all species to enable us to study the relative temporal positions of partly applicable events.

Table 3. Developmental sequences inferred for various species of *Niphargus*

Species	Inferred early events	Observed developmental sequence	Inapplicable events	Missing data
N. costozzae	(ABCD)	PHIL(NJG)FT(OQ)K	EMRS	ABCD
N. hebereri	–	(AB)(CDHJ)GP(KQ)	EFILMNORST	–
N. krameri	–	(BD)(ACI)HPGJ(FN)ELOTR (KS)Q	M	–
N. longicaudatus-Cres	L	D(IC)HPBNG(TR)(QK)S	AEFJMO	–
N. longicaudatus-Napoli	(CDL)	IBP(GT)FNQS(ER)K	AHJMO	–
N. longiflagellum	–	(AB)(CD)(EFL)I(KN)POQ	GHJMRST	–
N. salonitanus	(ABLMNO)	DCP(IH)	EFGJKRST	Q
N. spinulifemur	–	DB(ACI)GE(FH)PTRLKJQS	MNO	–
N. spoeckeri	(BCDIHGP)	LNFRTKOJ	AEMQS	BCDGHIP

Inferred early events: events that were not observed directly but inferred to have occurred in specimens younger than those present in our sample (e.g. in the embryo or in one of the earliest instars which were lacking in our samples). These events were given the lowest rank. In the case of multiple events, all events received the same, average rank.

Observed developmental sequence: the temporal occurrence of events as inferred by the consensus method of Velhagen (1997). Events in brackets occurred synchronously and, when ranked, received the same, average rank.

Inapplicable events: events that do not occur in the given species.

Missing data: events for which no data were available due to either lack of material (apart from those that could be inferred to be early events) or damaged material.

The premises for quantifying the extent of dissociation were as follows. We assume that events that are obligatorily associated (e.g. those that form a developmental module or are otherwise functionally/developmentally dependent upon one another) should stay in a similar relative timing to one another in the developmental sequences of all species. Moreover, the ranks of such events should be roughly similar across species (i.e. shifts of these events would be minimal). If large clusters of such events exist, phenotypic divergence (PD; Bininda-Emonds et al. 2003b) across species in this period of life should be minimal (or at least strongly reduced) with respect to other times. Two tests adapted from Bininda-Emonds et al. (2003b) were used to study the distribution of PD across the species. First, we calculated the mean rank of an event across species and examined the distribution of the variation of that mean. The expectation is that relatively stable events will possess a smaller standard deviation in their rank than do events that dissociate easily and evolve independently. Inapplicable events disturbed the analysis heavily, causing artificial early shifts of late events in shorter sequences (because the total number of ranks is reduced in such sequences). To lessen the influence of such events, we excluded those events that were absent in three or more species (events A, E, J, M, O, R, S; see Table 2) and repeated the analysis with this modified subset of the data.

For the second test, we subdivided the entire period of development into shorter subperiods (equal to 0.50, 0.33, 0.25 and 0.20 of the total developmental period) and recorded whether or not a given event occurred during that interval in a sliding-window analysis. With low PD, a given event will be mostly present or absent from all the species within a given period, whereas an event with a high PD will be about equally present and absent. We calculated PD with the metrics developed by Bininda-Emonds et al. (2003b). In short, overall PD of a given period is calculated as the average PD of all individual events as defined by $\mathrm{PD}_{\mathrm{event}} = 1-|(n_{\mathrm{p}}-n_{\mathrm{a}})/(n_{\mathrm{p}}+n_{\mathrm{a}})|$, where n_{p} and n_{a} are the numbers of species in a given period in which the focal event is present and absent, respectively. Because $\mathrm{PD}_{\mathrm{event}}$ is expressed as a relative number, it tends to be resistant to artifacts caused by inapplicable and missing data. Even so, because inapplicable events did not allow us to define temporal subperiods on the basis of ranks here (Bininda-Emonds et al. 2003b), we relied on standardized absolute timings instead (see under Section "Temporal component"). To diminish any artifacts arising from a single subjective starting placement of a subperiod on the entire sequence, we used two starting placements, shifted with respect to one another with a magnitude of half of that of the period duration (e.g. the first placement set of subperiod of size 0.33 used relative times 0.00, 0.33 and 0.66, whereas the second set used the times 0.17, 0.50 and 0.84).

Troglomorphic and sexually dimorphic features

Troglomorphic features, which include both reductive and constructive traits (Christiansen 2005), are generally considered to be more susceptible to convergence across species due to their strongly adaptive nature (Langecker 2000; Aden 2005; Christiansen 2005). However, Marques and Gnaspini (2001) emphasized the important fact that none of these features should be considered as homoplastic a priori, so that each could potentially provide substantial information on the phylogeny of subterranean animals. With our data we address the constructive morphological traits only, and then only in those species that differ the most in their degrees of troglomorphism: *N. longiflagellum*, *N. salonitanus* (both strongly troglomorphic) and both populations of *N. longicaudatus* (less troglomorphic). We focused on the respective elongation of antenna I and pereopod VII in particular (with the latter unfortunately not being observable in *N. salonitanus* because of the heavily damaged nature of the specimens). In adults of the different species, these appendages exhibit remarkably different relative lengths, which may be a consequence of differences in growth rate and/or a shift in the timing of the onset and/or offset of growth. To establish which mechanism(s) determine(s) constructive troglomorphic traits, we compared the growth rate of the trait, the shift in its time of offset, and its relative length in adults. Growth rate was quantified as the slope (coefficient) of the regression line for appendage length versus age, restricted to between the onset and offset points (similar results were obtained from log–log transformed plots). The offset point was inferred from the developmental sequence. In addition, the standardized absolute timing of growth offset was also compared between the species.

Some species exhibit rami of uropods I and III that are highly elongated in adult males (i.e. sexual dimorphism). "Elongated uropods" have been described by several authors (e.g. Stoch 1998), with the degree of sexual differentiation of either of the uropods forming an important taxonomic character for the group. However, it has never been investigated whether both uropods are elongated to the same extent in the sexually dimorphic species, nor when such elongation occurs (or is subject to heterochrony). Thus, we compared the growth rate, relative length in adults, and onset of elongation of each appendage, both in terms of standardized absolute timings as well as with respect to each other.

Results

Events and developmental sequences

The events studied are presented in Table 2, their order of appearance in each species is shown in Table 3.

Table 3 also attempts to distinguish whether missing events derive from being inapplicable in the species in question or were not observed due to damaged material or scarce samples. From Table 3, it is clear that the developmental sequences differ greatly among species, with no conserved subset of events maintaining its internal order of appearance across all species.

All species were characterized by at least one apparently inapplicable event, with *N. hebereri* having 10 such events. Most of the inapplicable events for a given species occur late in the developmental sequence of the other species in which they appear (Fig. 3A), with events that are inapplicable in a greater number of species (i.e. four, five or six) tending to occur in the second half of the developmental sequence. Most of the latter events also display a highly variable position (rank) in the developmental sequences for those species in which they occur (S.D. = 2.2–4.5; see Fig. 3A). By contrast, the more universal events, ones that were present in all species (four events) or absent in only one or three species (two and three events, respectively), were all found in the first half of the developmental sequence as determined from their average rank.

Plotting the standard deviation in the position of an event with respect to its average rank (Fig. 3B) suggests, albeit weakly, that late events might be subject to larger shifts in their relative timing (i.e. show more variability). However, a subset of the data excluding those events that were inapplicable the most frequently (and will therefore impact on the total lengths of the developmental sequences the most) showed instead that the relative timing of the peripheral events – the late ones as well as the early ones – was less variable than that of the middle events (Fig. 3C). These results must be viewed somewhat critically because (1) events that are on average closer to the ends of the developmental sequence tend to show reduced variation in their relative positions because they can move chiefly in one direction only ("edge effect" sensu Bininda-Emonds et al. 2003b); (2) the total lengths of developmental sequences, even with the most frequently inapplicable events removed, can still differ by up to two events, which might artifactually increase event variability in our relatively small event set, particularly for the later events and (3) events that were reconstructed as having to occur within a missing part of the developmental sequence were given an artificial rank that might not reflect their true position, especially when other events occur in the same missing portion of the sequence. The PD analysis, which avoids many of these potential artifacts including the edge effect, supports the general pattern found using the subset of the data in that, regardless of the window size employed, the highest phenotypic diversity tended to occur in the middle of the developmental sequences (Fig. 4). For example, with a window size of one-third of the total sequence length, initial PD values ranged from 0.22 to 0.33, before increasingly steeply during the first half of standardized absolute timing to a maximum of 0.46, followed by a decrease to values of 0.26–0.33 that are maintained throughout the remainder of the standardized absolute timing interval. For the two other window sizes, the highest PD was similarly found in either the second quintile (0.2–0.4; PD = 0.55) or the second quartile (0.25–0.5; PD = 0.54) of the standardized absolute timing.

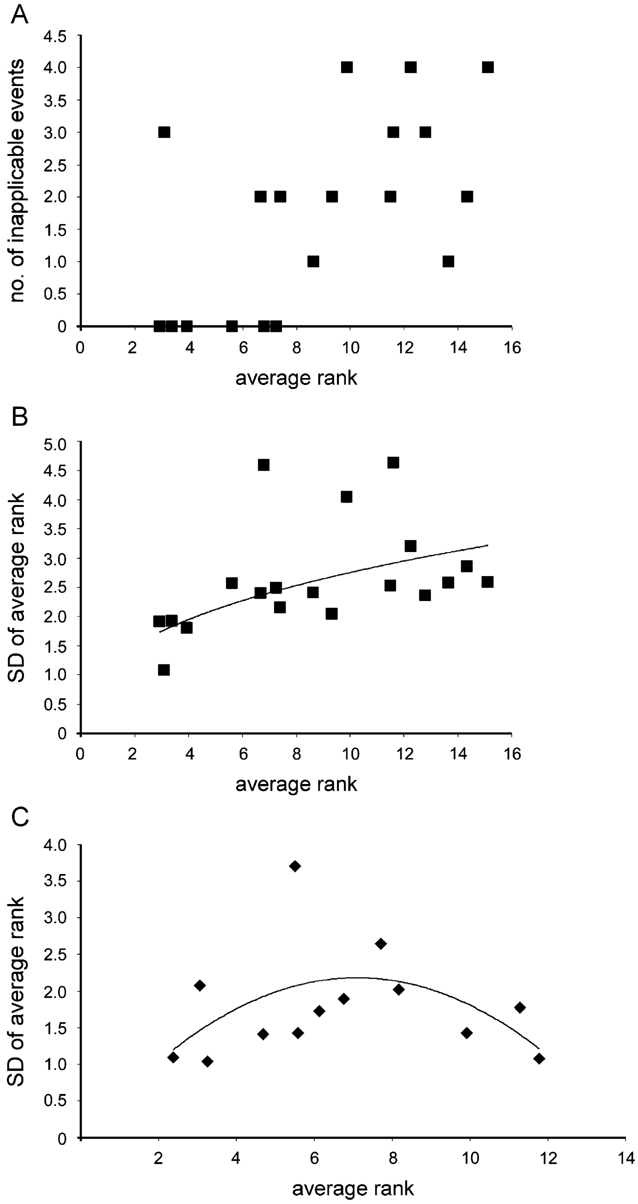

Fig. 3. Heterogeneity of developmental sequences. (A) Number of species for which an event was inapplicable versus average rank of the event as derived from species in which it occurred. (B) Variation in rank of all events. (C) Variation in rank with most frequently inapplicable events excluded.

Troglomorphic and sexually dimorphic features

The growth rates of antenna I and pereopod VII do not differ between populations of *N. longicaudatus*. However, a clear rate difference between either population and *N. salonitanus* or *N. longiflagellum* is evident for both appendages (Table 4) despite their relative and absolute timings occurring at only slightly different times (Tables 3 and 4). In all species for which data were available, the offset in the growth of pereopod VII occurs late, in either the fourth or the fifth quintile of the standardized absolute timing. More informative, however, is the development of antenna I. In both populations of *N. longicaudatus*, the offset of the relatively slow growth of this appendage is a late event. By contrast, the relatively early offset (third quintile) of the remarkably elongated antenna in *N. longiflagellum* suggests the need for an elongated appendage in subadult specimens. In *N. salonitanus*, antenna I never shows a decline in growth rate, even in some of the largest, fully developed specimens. Thus, the offset of growth of antenna I apparently coincides with the offset of growth of body length in this species.

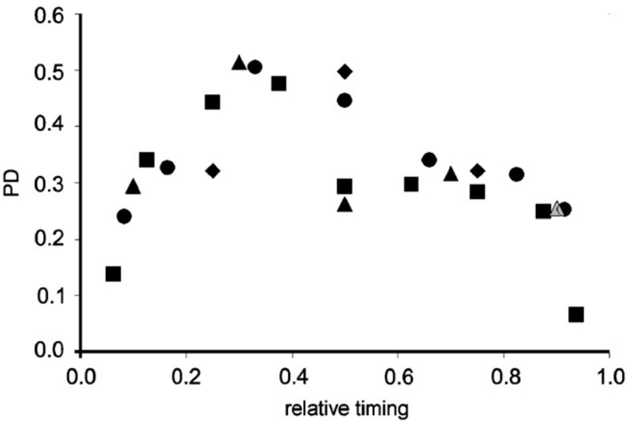

Fig. 4. Distribution of phenotypic diversity of events as a function of their position in the developmental sequence (normalized). Positions estimated by subdividing entire developmental sequence into subperiods of sizes 0.5 (diamonds), 0.33 (triangles), 0.25 (squares) and 0.2 (circles).

Five studied taxa (*N. longicaudatus*-Cres, *N. longicaudatus*-Napoli, *N. krameri*, *N. spinulifemur*, *N. spoeckeri*) possess elongated rami on both uropods I and III, with only the distal article of the exopodite of uropod III being elongated in one additional species (*N. costozzae*). There is little variation in the degree of elongation and growth rate of the endopodite of uropod I among

Table 4. Comparison of growth rates of the two troglomorphic features: antenna I (a I; above diagonal) and pereopod VII (pp VII; below diagonal)

Taxon		*N. longicaudatus*-Cres	*N. longicaudatus*-Napoli	*N. longiflagellum*	*N. salonitanus*
	a I pp VII	$k = 0.35$ rl = 0.3 at = 4	$k = 0.34$ rl = 0.33 at = 5	$k = 0.62$ rl = 0.6 at = 3	$k = 0.52$ rl = 0.53 –
N. longicaudatus-Cres	$k = 0.36$ rl = 0.29 at = 4		$p = 0.85$	$p = 0.002$*	$p = 0.056$
N. longicaudatus-Napoli	$k = 0.37$ rl = 0.33 at = 5	$p = 0.74$		$p = 0.002$*	$p = 0.009$*
N. longiflagellum	$k = 0.55$ rl = 0.53 at = 4	$p = 0.015$*	$p = 0.05$*		$p = 0.05$*
N. salonitanus	– rl = 0.53 –	–	–	–	

k = growth rate coefficient for antenna I and pereopod VII; reconstruction of k for pereopod VII in *N. salonitanus* was unreliable because of heavy damage to the specimens; rl = relative length of appendage in adult specimens; p = p-value testing null hypothesis of no difference between the pair of coefficients between two species (using test by Snedecor and Cochran 1976); at = absolute standardized timing, given as quintile in which event occurs.

*Difference is statistically significant at nominal alpha of 0.05.

Table 5. Comparison of growth rates of the two sexually dimorphic features: uropods I and III (u I, u III; below and above diagonal, respectively)

Taxon		*N. longic.*-Cres	*N. longic.*-Napoli	*N. krameri*	*N. spinulifemur*	*N. spoeckeri*	*N. costozzae*
	u III u I	$k = 0.19$ rl = 1 at = 2	$k = 0.16$ rl = 1 at = 3	$k = 0.19$ rl = 1 at = 2	$k = 0.221$ rl = 1 at = 2	$k = 0.1$ rl = 0.5 at = 3	$k = 0.38$ rl = 1 at = 3
N. longicaudatus-Cres	$k = 0.12$ at = 2		$p = 0.68$	$p = 0.9$	$p = 0.34$	$p = 0.005$*	$p = 0.045$*
N. longicaudatus-Napoli	$k = 0.13$ at = 4	$p = 0.95$		$p = 0.67$	$p = 0.39$	$p = 0.08$	$p = 0.29$
N. krameri	$k = 0.17$ at = 3	$p = 0.11$	$p = 0.75$		$p = 0.13$	$p = 0.0001$*	$p = 0.001$*
N. spinulifemur	$k = 0.11$ at = 3	$p = 0.7$	$p = 0.85$	$p = 0.09$		$p = 0.0001$*	$p = 0.008$*
N. spoeckeri	$k = 0.12$ at = 2	$p = 0.9$	$p = 0.93$	$p = 0.03$*	$p = 0.55$		$p = 0.002$*
N. costozzae	–	–	–	–	–	–	

k = growth rate coefficients for endopodite of uropod I and distal article of exopodite of uropod III; rl = approximate relative length of distal article of uropod III exopodite compared to proximal article in adult specimens; p = p-value testing null hypothesis of no significant difference between the pair of coefficients between two species (using test by Snedecor and Cochran 1976); at = absolute standardized timing, given as quintile in which event occurs.

*Difference is statistically significant at 0.05 level.

the above-mentioned species. However, the distal article of the exopodite of uropod III is strongly differentially elongated. In *N. spoeckeri*, the length of the distal article does not exceed one-half of the length of the proximal article. By contrast, its length approaches that of the proximal article in *N. costozzae*, *N. krameri*, *N. spinulifemur*, and both populations of *N. longicaudatus*. A significant difference in the growth rate of the distal article of the exopodite was also found between *N. spoeckeri* and *N. costozzae*, with respect to both remaining species as well as to one another. Otherwise, growth rates for these features did not differ significantly between *N. krameri*, *N. spinulifemur*, and both populations of *N. longicaudatus* (Table 5). The relative absolute timings and the relative order of the onset of growth in uropods I and III is even more variable (Table 3). In *N. krameri* and *N. spinulifemur*, the onset of elongation in uropod III just precedes that of uropod I. In *N. longicaudatus*-Napoli, the onset of elongation of uropod III is shifted to an earlier position with respect to other events, whereas both onset events occur simultaneously in *N. longicaudatus*-Cres. Finally, the onset of the elongation of uropod I uniquely precedes that of uropod III in *N. spoeckeri*.

Discussion

Merging allometric and sequence heterochrony

Translating growth processes for their incorporation into analyses of developmental sequences (and the sequence heterochronies they reveal) represents a novel approach in heterochrony studies that extends the possibilities for analyzing such data. In particular, distilling the entire growth curve down to discrete events representing the major shifts in growth greatly facilitated comparisons of growth rates and also elucidated the full complexity of some heterochronic changes successfully (see the following section and Tables 4 and 5). It also makes the ongoing debates about terminology (e.g. Reilly et al. 1997 versus Gould 2000) less important, consequently reducing the danger of misinterpretations among students of heterochrony (Klingenberg 1998). However, in judging the suitability of analyzing allometric heterochrony in an event-based framework, two issues need to be kept in mind.

First, the reduction of the growth curve to a series of events entails an inherent loss of information compared to the absolute timing data that cannot always be compensated for, especially in complex cases of heterochronic change. For example, the timing of the offset of growth of antenna I in both populations of *N. longicaudatus* shifts only one position in the developmental sequence relative to that of the offset of growth of the exopodite of uropod I, disguising that the size of the shift equals one-quarter of the entire standardized absolute timing interval (data not shown). Thus, analyzing shifts within the framework of the standardized absolute timing would place the offset of growth of antenna I into the fourth quintile (Table 4) or even into the third quartile (not shown). Moreover, several aspects of event analysis, such as comparisons of multiphase growth, remain unexplored or problematic (Klingenberg 1998). Finally, a fundamental limitation of encoding growth curves as discrete events is the accurate placement of the critical point. This placement depends heavily on the accuracy of the growth curve itself, which depends in turn on the number of specimens studied. As such, analyses of incomplete growth curves may lead to biased conclusions.

A second and perhaps even more difficult problem is that of establishing the homology of the events derived from the allometric data (Klingenberg 1998). Growth curves unquestionably represent the aggregated dynamics of a multitude of unknown developmental mechanisms that operate and interact at the organismal, tissue and cellular scales. Although it may be reasonable to assume that the elongation of any single, entire appendage is driven by the same underlying growth process (e.g. Gilbert and Bolker 2001), one should not assume a priori that the same regulatory mechanisms are operating across all species. The problem may be especially pronounced when complex shapes (e.g. propodi in this study) represent the objects of interest.

Similarly, and more fundamentally, we cannot always ensure the homology (identity) of specific characters across species, sometimes not even across individuals within a species. In the present study, a cogent example involves the various spines examined, concerning which we have no guarantee that we are always observing the development of the same structure. In this case, our observations indicate that the spines on the epimeral plates, urosomites and telson are not precisely defined temporally-spatially. Thus, even in fully developed specimens, the number of spines in topologically homologous groups can vary greatly within a population. In analyzing these structures, therefore, we need to assume that their development is a continuous process in which the spines differentiate from the underlying topologically homologous tissues, with their number as well as their overall development depending on mechanisms of onset, offset and rate regulation (e.g. Richardson and Oelschläger 2002). Other evolutionary processes in addition to heterochrony (e.g. heterometry; see Webster and Zelditch 2005) could also be analyzed within a similar framework of the onset-duration-offset of cellular mechanisms.

Phenotypic diversity, troglomorphoses and sexual dimorphism

On the basis of our data, it is not possible to reject the hypothesis that heterochronies, be they sequential or allometric, did not play any role in the evolution of *Niphargus*. Instead, heterochrony appears to have made an important contribution at least to the development of constructive troglomorphic traits, which in turn enabled the successful adaptation to and survival in subterranean waters.

A key property of the developmental sequences we studied is the comparatively high phenotypic diversity observed during the initial phase of postembryonic life. For comparison, the maximal PD values calculated for *Niphargus* compare favorably with or are higher than those from the early embryonic stages of vertebrates (0.54) or mammals (0.39; see Bininda-Emonds et al. 2003b). The high values we obtained for *Niphargus* might derive in part from pertaining to the postembryonic rather than the organogenetic period, the latter being widely held to be highly conserved among taxa (the phylotypic-stage hypothesis; see Bininda-Emonds et al. 2003b). However, the values are also derived from a much more taxonomically, and therefore presumably phenotypically, restrictive entity (genus versus class or higher). The apparently high degree of independence we infer as occurring up to the middle of the postembryonic period in *Niphargus* may serve as an important target for natural selection (Bininda-Emonds et al. 2003b). Certainly, the high degree of dissociation of the developmental events could serve as a potential, if partial, explanation for the notorious phenotypic variability in this genus that is normally attributed to numerous highly variable body parts being arranged in a 'mosaic' manner (e.g. Sket and Notenboom 1993).

The latter hypothesis confronts us with the following dilemma: to what extent is the 'mosaic' morphology of *Niphargus* (1) a legacy of high intraspecific genetic variability that, in turn, has resulted in a high degree of dissociation of developmental events on a population level and thus the extremely diverse morphological evolution of the group; versus (2) a consequence of the evolution of several independent lineages exposed to diverse selection forces in a variable environment. In attempting to answer this question, it is known that *Niphargus* generally has poor migratory abilities (Fišer et al. 2006b) but can adapt to extremely diverse habitats (Sket 1999a), thereby allowing several species to co-occur in the same restricted location (e.g. up to eight species in Vjetrenica cave, up to six interstitial species in a well close to Ljubljana city; Sket 1972, 2003).

Heterochrony might provide an important mechanism in this case to enable micro-niche partitioning (Denöel and Schabetsberger 2003) and thus a high local biodiversity. Answering the above dilemma satisfactorily, however, will require both a robust phylogenetic hypothesis for the genus and insights into intraspecific variation of developmental timing (see Reilly et al. 1997; Mabee et al. 2000) as well as the degree of genetic variation within the genus.

Another consequence of our observations is that the diversity of the genus may be even higher than previously thought if inapplicable events are viewed and discussed in light of 'reduction-novelty innovation' or 'homology-convergence'. Again, neither of these conjectures can be supported in the absence of phylogenetic information, but some evidence for hidden diversity (e.g. Mathieu et al. 1997; Lefébure et al. 2006) can be inferred from our data, especially for the troglomorphic features, to a lesser extent for the sexually dimorphic ones. For example, there appears to be a high degree of convergence in the morphology of the four appendages studied here, insofar as the different shifts inferred for growth rate and/or the timing of onset and/or offset (Tables 4 and 5) can result in the development of morphologically similar features. Support for this conjecture derives from indications that at least troglomorphic features emerge frequently and, from an evolutionary viewpoint, relatively quickly (compare Prevorčnik et al. 2004; Verovnik et al. 2005). Furthermore, it seems reasonable that either the sexually dimorphic or the troglomorphic elongation of appendages could have a simple genetic basis (Nijhout 1994; McNamara 1995; Klingenberg 1998; Smith 2003) and could result – at least for troglomorphic features – in differentiation on a population level (i.e. different ecophenotypes). In investigating all these issues, however, a crucial question is whether or not two structures with a common evolutionary origin remain homologous if their development differs (Gilbert and Bolker 2001).

The comparison of both populations of *N. longicaudatus* with *N. salonitanus* and *N. longiflagellum* revealed that constructive troglomorphic features emerge mainly through their accelerated growth. This seems reasonable, given that an elongated appendage might be needed throughout the life of the organism and not only in the adults. Despite the final relative lengths of the antennae in adult specimens of *N. longiflagellum* and *N. salonitanus* being similar, the observed differences in both their growth coefficients and their offset of growth suggest that the elongated antenna evolved independently in these species or, minimally, subject to two different mechanisms. For example, growth of the antenna in *N. longiflagellum* is extremely accelerated but halts relatively early. By contrast, growth is less accelerated in *N. salonitanus* but continues throughout the life of the organism.

The evolution of sexually dimorphic characters remains less clear and evokes many unanswered questions. Similar to the troglomorphisms discussed in the preceding paragraph, the less elongated uropod III of *N. spoeckeri* (as derived from a reduced growth rate in combination with its relatively late onset) possibly indicates its independent origin and/or different mechanisms underlying its development in this species. Likewise, the unique combination of an elongated uropod III and non-elongated uropod I in *N. costozzae* simultaneously requires a more intense growth of uropod III (Table 5) coupled with a reduced or unchanged growth of uropod I (depending on whether or not a non-elongated uropod is plesiomorphic), although a full evaluation is not possible in the absence of a phylogenetic framework.

The differences that we detected in the relative and absolute timings of the elongation of both uropods between the remaining four taxa (*N. krameri*, *N. spinulifemur*, and both populations of *N. longicaudatus*) cannot be evaluated unambiguously in the absence of a well-supported phylogenetic hypothesis. However, the differences in timing of the elongation of both uropods between the two populations of *N. longicaudatus* are comparatively large, which supports growing morphological (setae on gnathopods, spines on dactyls and telson) and molecular evidence (Trontelj et al. 2007) indicating that the populations represent two morphologically similar sibling species. This result strengthens our claim that the study of developmental processes might be a useful accessory tool for difficult taxonomic problems. In the case of *Niphargus*, the incorporation of developmental information might significantly refine future studies of putative 'cryptic' species to reveal the entire diversity of the genus.

Acknowledgments

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Appendix

List of possible heterochronies in *Niphargus*

Body length

Body length varies between 3 and 30 mm. However, changes in body size in which all proportions remain the same – proportional dwarfism and proportional gigantism (Gould 1977) – are extremely rare (examples are *N. pectinicauda* Sket, 1971 [3–5 mm]; *N. carcerarius* G. Karaman, 1989 [10 mm]; and *N. polymorphus* Fišer, Trontelj & Sket, 2006 [15 mm]). If we consider only body shape and ignore variability in appendages, several small (*N. kochianus* Bate, 1859 [5 mm]) and large species (*N. orcinus* Joseph, 1869 and *N. arbiter* G. Karaman, 1984 [up to 30 mm]) appear to be similar in general shape ('gestalt').

Head

Relative to body length, head length is larger in juvenile specimens than in older specimens (a general pattern). Paedomorphic large-bodied taxa with large heads include subspecies of *N. steueri* Schellenberg, 1935.

Body

Setae along the dorsoposterior margin of the pleonites increase in number during ontogeny and become thick and spine-like in some taxa. Paedomorphic species with four dorsoposterior setae include *N. elegans* Garbini, 1894, whereas peramorphic species such as *N. arbiter* have several dorsoposterior spine-like setae.

Similarly, the number of postero-dorso-lateral spine-like setae on urosomites I–II increase during development, but become thinner in adults. The number of setae is reduced (paedomorphic) in taxa like *N. aquilex* Schiödte, 1855; the setae become spine-like (peramorphic) in *N. arbiter*.

Epimeral plates are angular with concave or straight margins in juveniles. During development, they become increasingly subrounded, the ventral and posterior margin becomes convex, and the disto-ventral corner becomes indistinct, marked only with seta. Paedomorphic species: *N. orcinus*; peramorphic species: *N. aquilex*, *N. longicaudatus* Costa, 1851.

The telson has a highly variable shape that can change during the lifespan of some species and needs to be investigated further. Nevertheless, juveniles generally have longer spines than adults (e.g. *N. spoeckeri* (Schellenberg, 1933)). Paedomorphic long spines persist in some large (*N. lourensis* Fišer, Trontelj & Sket, 2006) as well as small (*N. forellii* Humbert, 1877) species. The latter species has extremely long telson spines, indicating that spines might elongate during development. In addition, the position of the lateral plumose setae changes during development in *N. arbiter*. Whereas the lateral plumosae setae are typically positioned medially, juvenile specimens of *N. arbiter* have these setae placed subapically, as do some paedomorphic members of the *jovanovici*-group (sensu Straškraba 1972) such as *N. multipennatus* Sket, 1956. In adults, these setae 'shift' proximally.

Antennae I and II

As noted in the text, antenna I varies in length. In addition, a change in proportions of the peduncular articles was noted in *N. arbiter*, in which a marsupial specimen had peduncular articles with relative lengths 1:0.8:0.45 (as in, e.g. *N. stygius* Schiödte, 1847), whereas in its mother the proportions were 1:1.1:0.7. Dissociation of growth also apparently occurs between articles 2 and 3. For instance, *N. steueri steueri* Schellenberg, 1935 has both articles elongated, in contrast to *N. steueri kolombatovici* S. Karaman, 1950, in which only article 2 is elongated.

Article 4 is longer than article 5 of the peduncle of antenna II – a pattern that may be reversed in some species or age classes. For example, article 4 of young specimens of *N. longiflagellum* S. Karaman, 1950 is longer than article 5, although the normal morphology is evident in adult specimens.

Mouthparts

Only few potential heterochronies were observed on the mouthparts. The number of setae on all mouthparts generally increases with age. However, in some fully developed members of large species, the number of these setae may be reduced: a 20 mm long specimen of *N. sanctinaumi* S. Karaman, 1943 has only two setae on the inner lobe of maxilla I, and *N. schellenbergi* S. Karaman, 1932 of similar length have only one flattened spine on the inner lobe of the maxilliped.

Coxal plates I–IV

Deep coxal plates in young specimens become shallower in adult specimens (e.g. *N. elegans*, *N. stygius podpecanus* S. Karaman, 1952). Peramorphic coxal plates are present in *N. aquilex* and *N. longicaudatus*, paedomorphic coxal plates in *N. pectinicauda* and *N. longiflagellum*.

Gnathopods I and II

The propods of the gnathopods of adult specimens of *N. kochianus* and *N. hebereri* have a (paedomorphic) shape similar to that in juvenile specimens of *N. spinulifemur* and *N. krameri*. By contrast, marsupial specimens of the *peramorphic N. arbiter* and *N. salonitanus* already show fully developed gnathopods.

Anterior setae on the dactyls of the gnathopods develop according to the following pattern (as observed in *N. costozzae*): a single seta → several single setae → several setae groups. Highly paedomorphic species (e.g. *N. kochianus*, *N. aquilex*) have a single seta only; the slightly less paedomorphic *N. orcinus* has several individual setae. Interestingly, the single seta observed in *N. multipennatus* is positioned slightly more distally than the one in *N. aquilex* (different topology!).

Dactyls on pereopods III–VII

The relative length of the dactylus is shorter in adult specimens than in juvenile ones (see text). Adult specimens of paedomorphic *N. forellii*, for example, have elongated dactyls. Furthermore, a spine with or without a seta can exist at the base of a nail. Numerous additional spines may appear on the dactyls, with the exact number varying between species, but generally increasing with the age of an individual. *Niphargus longicaudatus*, for example, has only one or two additional spines at most, whereas *N. valachicus* Dobreanu & Manolache, 1933 can have up to nine such spines.

Bases of pereopods V–VII

The bases are wide and convex-sided in young specimens, narrow and straight or concave-sided in adult ones (e.g. *N. s. podpecanus*). Distal lobes become progressively smaller during development. Paedomorphic species include *N. longiflagellum*, peramorphic species include *N. jovanovici* S. Karaman, 1931 and *N. croaticus* Jurinac, 1887.

Pereopod lengths

Only the length of pereopod VII was studied. Elongation of this leg proceeds to various degrees: in *N. aquilex* or *N. stygius podpecanus* (0.35–0.40 of body length) it is much shorter than in *N. dolichopus* Fišer, Trontelj & Sket, 2006 (1.00–1.10 of body length).

Uropod I

The rami of uropod I vary in both length and setal pattern. In *N. aquilex*, the endopodite is slightly shorter than the exopodite. In *N. longiflagellum*, *N. stygius*, and *N. longicaudatus*, the endopodites grow faster and exceed the length of the exopodite by factors of 1.0–1.1, 1.1–1.4 and >1.5, respectively. The number of setae on the endopodite in *N. longiflagellum* and *N. stygius* is equal to or slightly greater than that on the exopodite, with the length of the former element exceeding that of the latter only slightly. By contrast, the number of setae on the remarkably elongated endopodites in *N. longicaudatus* is higher than that on the exopodites.

Uropod III

The endopodite is remarkably elongated in some species, including *N. hebereri*. The exopodite is elongated in several species (but see text).

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