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1 1 **Developmental abnormalities in *Glomeris marginata* (Villers**
2 2 **1789) (Myriapoda:Diplopoda):**
3 3 **Implications for body axis determination in a myriapod**
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Abstract

Abnormally developing embryos (ADEs) of the common pill millipede *Glomeris marginata* have been investigated by means of nuclear staining and mRNA in situ hybridization. It showed that all ADEs represent cases of *Duplicitas posterior*, which means that the posterior body pole is duplicated. The severity of the duplication ranges from duplicated posterior trunk segments in one specimen to an almost completely duplicated specimen that only shares the very anterior head region. Remarkably, none of the encountered ADEs represents a case of *Duplicitas anterior* (duplicated anterior pole), or a case of *Duplicitas cruciata* (cruciate duplication with two anterior and two posterior poles). This observation is discussed in the light of earlier reports on *G. marginata* ADEs that claim to have found these abnormalities. The lack of any other axial abnormality aside from *D. posterior* implies that early axis determination in *G. marginata*, and possibly myriapods in general, underlies the developmental mechanisms that prevent the formation of any other type of axial duplication. It is proposed that the formation of *D. posterior* type embryos could be caused by the formation of two instead of only one posterior *cumulus* early during development.

59 **Introduction**

60 There has been a long tradition of study regarding abnormally developed specimens
61 in order to investigate early axis formation in arthropods. These studies date back to
62 long before the advent of modern genetic methods (reviewed in e.g. Sander 1988,
63 1997). Most of the specimens that have been investigated in these studies have been
64 the result of invasive maneuvers such as ablation, cutting and annealing, chemical
65 treatment, radiation, centrifugation, and strangulation of developing embryos (e.g.
66 Krause 1934, Schnetter 1934, Sekiguchi 1957, Yajima 1960, Kalthoff and Sander
67 1968, Itow and Sekiguchi 1979). Work on insects has initially been interpreted as
68 supporting the mosaic development model. Later, general thought shifted in favor of
69 regulatory centers, and finally the gradient theory is now generally accepted
70 (reviewed in Sander 1997). Although the majority of these ontogenetic studies have
71 been conducted on insects, chelicerates have also been studied extensively during this
72 time (e.g. Holm 1952, Ehn 1962, 1963, Juberthie 1968, Seitz 1970). Crustaceans are
73 less thoroughly investigated in this respect and relatively few studies exist reporting
74 on similar abnormally developed specimens (Chatton 1909, Harzsch et al. 2000,
75 Harlioglu 2002, Alwes and Scholtz 2005). Within the Myriapoda, the majority of data
76 are known from centipedes (e.g. Selbie 1913, Juberthie-Jupeau 1962, Hubert 1968,
77 Minelli and Pasqual 1986, Kettle et al. 1999, Lesniewska et al. 2009). Relatively few
78 studies deal with progoneate myriapods such as diplopods (Balazuc and Schubart
79 1962 (and references therein), Juberthie-Jupeau 1968, 1969, 1974, Hubert 1968,
80 Ceuca 1989). The abnormally developed myriapod specimens have in all cases been
81 the results of temperature shock treatment or have occurred naturally. A number of
82 reports on diplopod abnormalities come from the pill millipede *Glomeris marginata*

(Villers 1789) (Hubert 1968, Juberthie-Jupeau 1968, 1969, 1974). The early development of this species is sensitive to temperature changes, and temperature shocks often lead to germ band duplications. Occurrence of nymphs with duplicated posterior germ bands (*Duplicitas posterior*), duplicated anterior germ bands (*Duplicitas anterior*), and cross-wise duplications of the germ band (*Duplicitas cruciata*) have been reported previously (Juberthie-Jupeau 1962, 1968, 1974, Hubert 1968). Reports of abnormally developed embryonic specimens, however, are scarce. One exception is the recently published communication on a *G. marginata* embryo that reports missing dorsal segmental derivatives (Janssen 2011a).

The abnormally developing *G. marginata* embryos (ADEs) that are described in this paper were collected under controlled laboratory conditions (20-21°C). This is because abnormally developing embryos under natural conditions are rare (Hubert 1968). All ADEs described in this paper are of the type *D. posterior*. This may contradict earlier work that states the occurrence of *D. anterior*, *D. cruciata* and *D. completa* type germ band duplications in the same species (Juberthie-Jupeau 1968, 1969). This hypothesis is therefore carefully re-evaluated and discussed. Uncertainties in identifying morphological structures such as limbs and segments in ADEs have been addressed in three ways: firstly by the choice of embryonic versus nymphal specimens, the analysis of subsequent (abnormal) developmental stages, and finally the use of differentially expressed genetic markers.

Material and Methods

Animal husbandry and abnormally developing embryos

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107 Mature specimens of *G. marginata* were collected annually (February/March 2003 to
108 2010) in the Reichswald forest near the city of Kleve (Nordrhein-Westfalen,
109 Germany). They were cultivated in plastic containers (22 cm x 13 cm x 5 cm) filled
110 with decomposing beech leaves (food) and moist clay (building material for egg-
111 chambers) at a constant temperature of 21-22°C. Females laid eggs for 3-4 weeks
112 after they were collected. Six to eleven days after oviposition eggs were removed
113 from the egg-chambers by hand. After six days of development at 21-22 °C the
114 blastoderm has formed (stage 0) and after eleven days the embryo is rolled up and the
115 cuticle starts to form (stage 6.1) (staging after Janssen et al. (2004)). Embryos were
116 fixed for 4 hours in 1 ml 4% formaldehyde in phosphate buffered saline (PBS) pH 7.4
117 with 0.1% Tween-20 (PBST) and 1ml heptane. After that treatment embryos were
118 stored in methanol at -20°C pending further examination. ADEs of individuals older
119 than approximately stage 2 were identified under a dissecting microscope; younger
120 stages were not considered because they do not possess well-developed
121 morphological landmarks. The ADEs were stored separately and simultaneously
122 stained for the investigations reported in this work.

123 124 *Gene cloning and probe synthesis*

125 RNA isolation and cDNA synthesis were performed as per Janssen et al. (2004). A
126 fragment of the *Glomeris* ortholog of *spineless* (*ss*) was amplified via RT-PCR with
127 the degenerate primers *ss_fw1* (ACN TGY GAN GGN GAT GTN TTY T) and
128 *ss_bw1* (TTR AAR TCC ATN GTN CKY TTN CC) in a first reaction, and *ss_fw2*
129 (TGY ACN CCN TTY GGN CCN CC) and *ss_bw1* in a semi-nested PCR reaction.
130 The fragment was cloned into a plasmid vector (pCRII-TOPO, Invitrogen).

131 A fragment of *six-3/optix* was amplified from *Glomeris* with the degenerate primers
 132 *six3_fw* (CCN GTN GCN CAY CCN AAY TG) and *six3_T7* (=bw)
 133 (TAATACGACTCACTATAG AAC CAR TTN CCN ACY TGN GTN GG). The
 134 isolated fragments will herein be referred to as *ss* and *six3* respectively.
 135 Sequences of the two gene fragments were determined by sequencing (Big Dye
 136 Terminator Cycle Sequencing Kit; Perkin-Elmer Applied Biosystems, Foster City,
 137 CA, USA) chemistry on an automatic analyzer (ABI3730XL; Perkin-Elmer Applied
 138 Biosystems) by a commercial sequencing service (Macrogen, Seoul, Korea).
 139 Sequences are available in GenBank under the accession numbers HE974354 (*Gm-*
 140 *ss*), and HE974353 (*Gm-six3*).
 141 The PCR fragment of *six3* was purified using a QIAquick PCR purification kit
 142 (QIAGEN). This fragment was then used, without cloning into a vector, as template
 143 for subsequent RNA-probe synthesis (David and Wedlich 2001). In both cases, *ss* and
 144 *six3*, DIG-labeled RNA probes were synthesized with T7 RNA polymerase
 145 (ROCHE). Probes were purified with the RNeasy Mini kit (QIAGEN) prior to whole
 146 mount in situ hybridization.
 147
 148 *Whole mount in situ hybridization and nuclear staining*
 149 Cell nuclei were visualized via incubation of the embryos in 1 µg/ml of the
 150 fluorescent dye 4-6-Diamidin-2-phenylindol (DAPI) in PBST for 30 minutes followed
 151 by subsequent washes in PBST.
 152 Whole mount in situ hybridization (WISH) was performed as per Janssen et al.
 153 (2011). In order to choose appropriate genetic markers for the later investigation of
 154 ADEs, the embryos were re-hydrated with PBST, DAPI-stained, and photographed,
 155 prior to WISH. To avoid possible decomposition of DAPI-treated embryos stored in

phosphate buffer, they were subsequently dehydrated in a methanol series and stored at -20°C in 100% methanol for up to several weeks before the WISH procedure was performed. Possibly because of this additional hydration/dehydration-step the WISH did not work as expected under standard conditions. Information on gene expression patterns used to identify morphological structures in ADEs is provided as an electronic supplement.

Documentation

Imaging of stained embryos was performed with a digital camera (Axiocam; Zeiss, Jena, Germany) attached to a dissection microscope (Leica, Heerbrugg, Switzerland). Pictures of the DAPI treated embryos were taken under ultraviolet light. Brightness, contrast and color values were, when needed, corrected using image-processing software (Adobe Photoshop CS2, V.0.1 for Apple Macintosh; Adobe Systems Inc. San Jose, CA, USA).

Results

Normal development

It may be useful to shortly recapitulate normal development of *G. marginata* in order to better understand the abnormalities described in this paper. From stage 0 (blastoderm stage) to stage 2 the anterior segments including the first trunk segment (T1) form from the *regio germinalis*, and T2 and T3 split off from the posterior segment addition zone (SAZ) (Janssen et al. 2004). At stage 3, the limb buds of the walking legs appear on T1 to T3, and dorsal tissue corresponding to the anterior trunk segments develops (Fig. 1A). At stage 4, the limb buds on T4 appear (Fig. 1B).

Throughout embryogenesis the limb buds on T1-T3 will remain notably better developed than those on T4-T8. The premandibular segment and the postmaxillary segment are without appendages (Fig. 1B). At stage 5 the first embryonic diplosegment (T5+T6) forms by dorsal fusion (Janssen 2011b). The head forms a morphological unit, and the germ band begins to bend in. At this point the labrum is clearly visible and covers the mouth (Fig. 1C). At stage 6 the germ band is fully bent, anterior and posterior ends come into close contact (Fig. 1D). Dorsal segmental tissue further extends dorsally and at subsequent stages will meet during the process of dorsal closure (not shown). More detailed staging of *G. marginata* embryos is described in Dohle (1964) and Janssen et al. (2004).

Abnormally developing embryos

All abnormally developing embryos (ADEs) represent cases of *Duplicitas posterior*. That is, the embryo possesses two posterior poles that share a single anterior pole. These ADEs can be subdivided into four classes, described below. A detailed description of each individual ADE is provided as an electronic supplement. The data are briefly summarized in Table 1.

Class-I: Cases of *Duplicitas posterior* with symmetrically duplicated posterior germ bands

Embryos E01 to E10 are characterized by successively less shared anterior tissue (i.e. the largest amount of shared anterior tissue is observed in E01 (Fig. 2A), the smallest amount of shared anterior tissue can be seen in E10 (Fig. 2J). The two posterior germ bands are equally well developed. The single non-duplicated anterior pole of each embryo is well developed. Fusion of morphological structures, such as limbs and

dorsal segmental tissue, occurs in the contact zone between the two posterior germ bands and the single anterior portion of the embryo (Figs 2 and supplementary figure Fig. S2).

Class-II: Cases of *Duplicitas posterior* with asymmetrically duplicated posterior germ bands

In ADEs with asymmetrically duplicated posterior germ bands (E11-E14, E24 and E25), one embryo (major) is almost normally developed, whilst the other (minor) germ band is fused with its “anterior” portion to the anterior trunk region of the major germ band. In all cases the minor germ band is fused with the major germ band, and never represents a headless solitary embryo (Fig. 3 and supplementary figure Fig. S2). The minor germ band is in all cases dorso-ventrally compressed and part of the ventral tissue appears to be missing. As a result, the limb buds of the minor germ band stand very close together (or are even fused). In normally developing embryos this is the area where the ventral nervous system is located (cf. Figs 1).

Class-III: Cases of *Duplicitas posterior* - separate germ bands without anterior head segments

In two ADEs (E15 and E16) two separate (not fused) posterior germ bands that lack anterior tissue form (Fig. 4). The germ bands face each other with their “anterior” poles, but are located parallel to each other. The anterior most extension of the two germ bands is at approximately the same level as the equator of the developing egg. In both germ bands of E15 (marked (a) and (b)) the mandibular segment represents the most anterior tissue (Fig. 4A-C), and only one mandibular bud has developed (see expression of *cnc*). In both germ bands of E16, the pmx-segment represents the anterior-most segment that is clearly identifiable (see expression of *Antp* as a marker for T1) (Fig. 4D-F).

231 Class-IV: Cases of *Duplicitas posterior* - fused germ bands without anterior head
232 segments

233 Four developing eggs (E17-E20) were identified in which the two germ bands are
234 fused with their anterior portion. In these embryos the most anterior head segments
235 appear to be missing (Fig. 5). In all cases the mandibular segment is the most anterior
236 and identifiable segment. Tissue of unclear affiliation is located anterior to this
237 segment.

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240 **Discussion**

241 *All germ band duplications in G. marginata represent bona fide cases of Duplicitas*
242 *posterior - the result of a developmental constraint?*

243 A number of studies report on germ band duplications in *G. marginata* (Dohle 1964,
244 Hubert 1968, Juberthie-Jupeau 1968, 1969, 1974) and other myriapods (a symphylan
245 and a centipede) (Selbie 1913, Juberthie-Jupeau 1961). Thus these duplication events
246 appear to be a frequently occurring phenomenon, even under natural conditions.

247 While the occurrence of duplicated posterior regions (*D. posterior*) is frequently
248 reported, only two papers describe the rare occurrence of *G. marginata* specimens
249 with duplicated anterior poles (*D. anterior*) (Juberthie-Jupeau 1968, 1969), and only
250 one paper reports a crosswise duplicated embryo (*D. cruciata*) (Juberthie-Jupeau
251 1968). However, no photographs are provided in these reports to unambiguously
252 prove the findings of *G. marginata* ADEs of the type *D. anterior* and *D. cruciata*.

253 Only a sketch is shown for the *D. cruciata* specimen (Juberthie-Jupeau 1968). This
254 sketch pictures the ‘cruciate’ specimen from a dorsal view and with fused anterior
255 heads, two pairs of antennae and two pairs of eyes (eye fields). This specimen

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256 represents the first nymphal stage (Juberthie-Jupeau 1968). Among the embryos
257 presented in this paper, E21 is most similar to the sketch provided in Juberthie-Jupeau
258 (1968). It is possible that this embryo would appear at later stages to be a morph
259 similar to that described as *D. cruciata*. Such a specimen would not represent a case
260 of *D. cruciata*, because it shares at least the anterior-most head region and does not
261 possess two free anterior poles and two free posterior poles as described for cases of
262 *D. cruciata* in other arthropods (cf. e.g. Brauer 1917, Holm 1952, Krause and Krause
263 1954). It is thus possible that the alleged *D. cruciata* embryo represents a
264 misinterpreted *D. posterior* embryo with minimal amount of shared anterior tissue.
265 Juberthie-Jupeau (1969) also described three specimens with either two free heads
266 (two specimens) or two dorsally fused heads (one specimen). She interpreted these
267 nymphs as cases of *D. completa* (Juberthie-Jupeau 1969). It is, however, likely that
268 these specimens indeed share the anterior-most tissue and that the head only appears
269 to be free after the rearrangement of the anterior portion of the head. Late during
270 ontogenesis the labrum and mouth move into a more posterior and lateral position,
271 while the eyes move dorso-laterally. As a result, initially shared anterior tissue will in
272 a later developmental stage, such as the nymphal stage, end up in a more posterior
273 position. Consequently, the forming head would appear to be free and without any
274 shared tissue.
275 Juberthie-Jupeau's claim that she discovered cases of *D. anterior* (Juberthie-Jupeau
276 1968, 1969) may rest on similar misinterpretations, although this is less likely given
277 that in a possibly misinterpreted *D. posterior* specimen the two posterior poles would
278 be easily identifiable. However, she described one of these specimens with a single
279 posterior pole but a significantly broadened anus (Juberthie-Jupeau 1969). It is
280 possible that this represents a *D. posterior* type specimen with minimal amount of

shared anterior tissue (in the opposing position) in which the posterior poles are secondarily fused, resulting in the unnaturally broadened anus. This may happen as a result of the posterior germ bands growing towards each other late during ontogenesis. The embryos E21 and E22 (Fig. S2A-D) may exemplify this scenario. At even later stages (nymphs) these embryos may appear to match the description of *D. anterior*.

Although the present study reports on only 25 specimens, more specimens with axial duplications have been observed, but not described here as they represent the same stages and abnormalities as the shown specimens. Altogether 63 specimens were found that all represent cases of *D. posterior*. This work is based on these 63 specimens. More *D. posterior* ADEs have been observed in the last years, but were not further investigated. These ADEs also represent exclusively cases of *D. posterior*. This corresponds with Dohle's (1964) statement that he found several specimens with duplicated posterior ends, but never a single specimen with duplicated anterior poles. It thus appears that all confirmed axial duplications reported so far for any myriapod species represent bona fide cases of *D. posterior*. This finding raises the question whether there may be an underlying developmental constraint that prevents the (natural) occurrence of any other axial duplication.

The phenomenon of head and trunk(s) at a right angle - a result of "lateral closure"

It appears in some cases that anterior and posterior portions of the ADE stand at a right angle to each other. In symmetrically duplicated and opposing germ bands with only a small amount of common anterior tissue, this seems to regularly be the case (Fig. 2E,G-I). The simple explanation for this interesting phenomenon is that the

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306 dorsal sides of two different germ bands fuse irregularly when the dorsal tissue
307 extends over the yolk. In normal cases the two dorsal edges of the same (single) germ
308 band fuse in this way during the process of dorsal closure. In embryos with duplicated
309 posterior germ bands, however, this possibility is excluded, because of spatial
310 limitations. The supernumerary germ band prevents two dorsal rims of the same germ
311 band to fuse, because it is physically in the way. Instead, the right side of one germ
312 band fuses with the left side of the other germ band (and vice versa) in a lateral
313 position (“lateral closure”). As a result of this miss-fusion, the suture of “lateral
314 closure” lies at right angle to the symmetry axis of the germ bands, and does not (as in
315 normally developing embryos) share the same axis (Fig. S3). This scenario has also
316 been observed and described by Juberthie-Jupeau (1969).

317

318

319 *Missing anterior tissue in D. posterior-type ADEs*

320 Two classes of ADEs have been described here that deal with twinned embryos
321 lacking the most anterior portion(s) of their germ bands (Figs. 4 and 5). The first class
322 (Class-III) describes specimens with parallel shifted germ bands (Fig. 4). The second
323 class (Class-IV) comprise of fused specimens (Fig. 5).

324 Two similar specimens are described in the literature. Hubert (1968) described one
325 first-stage nymph and one second-stage nymph of *G. marginata* that resemble the
326 specimens presented in this paper. Unfortunately, again, the description is incomplete
327 and solely relies on hand drawings. Hubert (1968) interprets a broad (fused?) flap-like
328 structure at the anterior contact zone of the two germ bands as the antennal rudiment.
329 This interpretation is supported by the presence of antennae-specific structures on the
330 tip of this appendicular structure (Hubert 1968). However, the presence of mandibles

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331 and maxillae in this specimen is not described or mentioned. It thus appears possible
332 that Hubert miss-interpreted fused mandibles as an antennal rudiment. Following this
333 interpretation, she describes another antennal rudiment in a stage-two nymph (Hubert
334 1968). For this specimen she actually presents a close-up drawing of the ventral side
335 of the nymph that shows a set of mandibles and a gnathochilarium (the maxillae). The
336 single ‘antennal rudiment’, however, strikingly resembles a walking limb. A possible
337 explanation as to why she may have refused to interpret this structure as a walking
338 limb may be that the (normal) full complement of eight limb-bearing segments (=16
339 walking limbs) is present in each of the two germ bands. This would make the single
340 structure (interpreted as a rudimentary antenna) unlikely to be a walking limb.
341 Juberthie-Jupeau (1974), however, showed that the number of trunk segments (and
342 thus walking limbs) in heat-induced ADEs of *G. marginata* may vary up to ten
343 instead of eight segments. The latter is the normal number of trunk segments in first
344 and second-stage nymphs (e.g. Enghoff et al. 1993).
345 The data provided in this paper, and the data reported by Hubert (1968) suggest that
346 in all cases the tissue anterior to the mandibles is lost or rudimentary in the ‘headless
347 twin’-type ADEs. This indicates that the interface between the mandibular and the
348 premandibular segment could possibly represent a special center of organization or
349 embryonic border. Tissue posterior to this border may be less prone to disturbances
350 during ontogeny.
351 Interestingly, missing anterior heads were never observed in embryos that do not
352 concurrently represent cases of *Duplicitas posterior*. This suggests a functional
353 connection between the presence of twinned posterior germ bands and the lack of the
354 anterior-most tissue.

356

357 *A theoretical explanation for the formation of D. posterior type ADEs*

358 The first morphological sign of the forming germ band is the *cumulus* that develops at
359 approximately 7-8 days (at approximately 18°C (Dohle 1964)), or 6-7 days after
360 fertilization (at approximately 20°C (Janssen et al. 2004, 2011)). Prior to that the
361 developing embryo represents a classical blastoderm (Dohle 1964). The *cumulus* itself
362 is recognizable as an area of greater cell density. It marks the vegetative (or posterior)
363 pole of the developing embryo. Occurrence of a *cumulus* has also been reported for
364 other myriapods (Heymons 1897, 1901). This ‘structure’ will later transform into the
365 SAZ from which new segments are specified and added to the extending germ band.
366 The *cumulus* represents the posterior pole of the embryo, and that appears to be of
367 special interest for the formation of *D. posterior* ADEs. The simplest way to explain
368 the formation of germ bands with duplicated posterior poles is to assume a duplication
369 of the *cumulus*. The experiments by Juberthie-Jupeau (1968, 1969, 1974) showed that
370 temperature shocks often lead to abnormal development of the type *D. posterior*. This
371 happens with the highest frequency in eggs that are around 3-4 days old, when the
372 three-day long temperature shock (Juberthie-Jupeau 1974) is applied. This represents
373 a time period from shortly before the formation of the blastoderm until the blastoderm
374 and the *cumulus* have formed (Dohle 1964). It is possible that the relatively high
375 temperature disrupts cell movements in the developing embryo. In this way *cumulus*-
376 forming cells (or *cumulus* founder cells) may be separated and/or moved to two
377 separate locations - the assumed two later forming *cumuli*.

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380 *Future perspectives*

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381 Some aspects concerning the presented data are still unclear, thus it would be
382 beneficial to further investigate ADEs to unambiguously clarify these points. For
383 example, the statement that ADEs of the type *D. anterior*, *D. completa* and *D.*
384 *cruciata* may not occur in *G. marginata* is solely based on negative results (i.e. the
385 absence of such ADEs from the samples underlying this and previous reports) and
386 seemingly contradicted earlier reports (discussed above) (Juberthie-Jupeau 1968,
387 1969). It is therefore planned to produce a much larger sample of ADEs by means of
388 temperature shocks as described by Juberthie-Jupeau (1968). This should either
389 produce a statistically relevant number of ADEs with exclusively posterior
390 duplications, to exclude any other kind of duplication, or indeed produce rare cases of
391 *D. anterior*, *D. cruciata* and *D. completa*. The examination of older stages (nymphs)
392 is planned in order to further understand how the morphology of the *D. posterior*
393 embryos changes over time. Such data may reveal whether the described *D. cruciata*
394 and *D. completa* embryos (Juberthie-Jupeau 1968, 1969) may in fact represent miss-
395 interpreted *D. posterior* embryos. It is also planned to further investigate ADEs by
396 means of molecular marker genes in order to gain further insight into how the critical
397 anterior tissue fuses/behaves in ADEs. Earlier embryonic stages may be of great
398 interest, in particular because they allow a two-dimensional view of the anterior head
399 segments prior to the formation of three-dimensional structures and before the
400 morphological rearrangement of the head begins (e.g. relocation of the position of the
401 mouth, regulatory events, and disturbances caused by the clashing of the two posterior
402 germ bands in the attempt to properly roll in and grow dorsally over the yolk).
403 Finally, detection of cell death via terminal deoxynucleotidyl transferase dUTP nick
404 end labeling (TUNEL assay) is planned for embryos with rudimentary or no anterior
405 head. This will reveal to what extent cell death is involved in these peculiar ADEs.

406

407

408 **Conclusions**

409 Four classes of ADEs can be distinguished in *G. marginata*: 1) symmetrically
410 duplicated germ bands that share a single anterior pole (the grade of duplication varies
411 from embryo to embryo), 2) asymmetrically duplicated germ bands that share a single
412 anterior pole; the anterior pole is clearly associated with one of the two posterior
413 poles (major); the minor posterior germ band is laterally fused with the major germ
414 band, 3) duplicated posterior germ bands without anterior pole; the two germ bands
415 are not fused; 4) duplicated posterior germ bands with a rudimentary anterior pole; the
416 two germ bands are fused. All classes represent variations of *Duplicitas posterior*.
417 That means that these embryos possess two posterior poles, but only one (shared)
418 anterior pole.

419 Other germ band duplications such as *Duplicitas anterior* and *Duplicitas cruciata* that
420 were reported previously were not found.

421 A probable reason for the occurrence of *D. posterior* type ADEs is the formation of
422 two posterior organization centers (*cumuli*) instead of only one, as the result of early
423 development at excessive temperatures.

424

425

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566 **Figure Legends**

567

568 **Fig.1 Normally developing embryos of *G. marginata*.**

569 In all panels anterior is to the left. Outgrowing limb buds of T1 to T3 are marked with
570 arrows in (A). The arrow in (B) points to outgrowing limb buds in T4. The bracket in
571 (C) marks the first diplosegment. Abbreviations: A, anterior pole; an, antennal
572 segment; av, anal valves; e, eye grooves; md, mandibular segment; mx, maxillary
573 segment; oc, ocular region; P, posterior pole; pmd, premandibular segment; pmx,
574 postmaxillary segment; S, stomodaeum; SAZ, segment addition zone; T1 to T4, first
575 to fourth walking limb bearing segment.

576

577 **Fig.2 Symmetrical ADEs of the type *Duplicitas posterior*.**

578 DAPI stained embryos (A-I) and corresponding bright field photographs (A'-I'). In all
579 panels anterior is to the left. Note that embryos in bright field and corresponding
580 DAPI panels are not orientated exactly in the same position, but in similar position.

581 Arrow in (A/A') points to the single shared T3 limb-bud; arrowheads point to
 582 corresponding T3 limb-buds. Asterisks in (B), and arrowheads and arrow in (B') mark
 583 maxillae. Arrowheads and arrow in (C) mark maxillae; filled circle indicates the
 584 position of a mandible. (c) shows a magnification of the anterior fusion-area of the
 585 embryo shown in (C). Asterisks in (D) and arrowheads and arrow in (D') mark
 586 mandibles. Asterisks in (E) and arrowheads and arrow in (E') mark mandibles.
 587 Asterisks in (F) and arrowheads and arrow in (F') indicate the position of antennae.
 588 Arrowheads in (F) point to putative tentorial openings in the pmd segment. Asterisks
 589 in (G) and arrowheads and arrow in (G') mark antennae. Asterisk in (H) and
 590 arrowheads and arrow in (H') mark antennae. Black asterisks in (H') mark two
 591 domains of *ss*-expression in the single (fused) antenna. Asterisks in (I) and arrows in
 592 (I') mark labral buds. Arrowheads in (I) point to artificial indentations that are most
 593 likely a result of the fusion process. Asterisk in (J) and arrowheads and arrow in (J')
 594 point to eye grooves. Abbreviations are as in Fig.1, and lr, labrum.

Fig.3 Asymmetrical ADEs of the type *Duplicitas posterior*.

597 All embryos are oriented with anterior to the left. Bright field photographs (A'-C')
 598 show same embryos as DAPI-stained embryos in (A-C). Ventral view on all embryos,
 599 except in panel (E) (lateral view). Arrowheads in (A/A' and C/C') mark antennae and
 600 maxillae. Asterisks in (A/A' - C/C') mark most anterior single limb bud of minor
 601 germ bands. Arrowheads in (A/B) indicate uncertain affiliation of the structure.
 602 Abbreviations as in Fig. 1, and P1, posterior pole of major germ band; P2, posterior
 603 pole of minor germ band.

Fig.4 Cases of *Duplicitas posterior* with separate germ bands and without anterior head segments

Images of DAPI-stained embryos (A-F) show same embryos in similar position as presented in bright field photographs (A'-F'). Arrows in (A) and (D) point to possible connective tissue. Question marks in (A/A') indicate tissue of unclear affiliation. Arrowhead in (B/B') marks single maxilla. Abbreviations as in Fig. 1, and (a) and (b) mark the two similarly developed germ bands.

Fig.5 Cases of *Duplicitas posterior* with fused germ bands and without anterior head segments

Left arrow in (A) marks a maxilla of germ band (a). Right arrow in (A) marks a limb bud of unclear affiliation. Arrowhead in (A/B) points to (possibly fused) mandibles. Question mark in (B) indicates tissue of unclear affiliation. Arrowheads in (C) mark fused maxillae and mandibles (cf. (E)). (D) Backside of the embryo shown in panel (C). Arrow in (F) points to anterior tissue of unclear affiliation. Asterisk in (F) marks the fused mandibles (cf. (H/h)). (G) Backside of the embryo shown in panel (F). Arrow in (I) points to tissue of unclear affiliation anterior to the fused mandibles. Asterisks in (I/J) mark abnormally developed posterior poles. Abbreviations as in Fig. 1, and (a) and (b) mark the two equally developed germ bands shown in panel (A).

Table Legend

Table 1 Summary of reported results on abnormally developed *G. marginata* embryos.

Abbreviations: an, antennal segment; *Antp*, *Antennapedia* gene; *cnc*, *cap-n-collar* gene; *Dll*, *Distal-less* gene; e, eye groove; *en*, *engrailed* gene; lr, labrum; md,

630 mandibular segment; mx, maxillary segment; pmx, postmaxillary segment; pre-an,
631 pre-antennal region; *ss*, *spineless* gene; T3, third walking limb bearing segment.

Figure

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No.	Stage	Shared Anterior Tissue (including)	Detected mRNA	Specific Remarks (in-detail description in supplement)	Figure(s)
Class-I: Cases of <i>Duplicitas posterior</i> with symmetrically duplicated posterior germ bands					
E01	3	T3	<i>Dll</i>	largest amount of shared anterior tissue; three T3 limb buds	2A/A'
E02	5	(pmx?), mx	<i>Dll</i>	inner limbs of T1 in close proximity	2B/B'
E03	4.1	md	---	inner maxillae in close proximity; three md buds	2C/c
E04	5	md	<i>six3+ss</i>	three md buds	2D/D'
E05	4	md	<i>ss</i>	three md buds; tissue anterior of an (possibly remnants of lr, oc and S)	2E/E'
E06	3	md/an	<i>ss</i>	three md buds; three an buds; remnants of two pmd segments	2F/F'
E07	4	an	<i>cnc+ss</i>	md segments abutting each other; three an buds, S and two an buds on one side, third an bud on other side of embryo; tissue “anterior” to an buds on either side (putative remnants of lr and oc)	2G/G'
E08	4.1	an	<i>ss</i>	three an buds	2H/H'
E09	4	lr	<i>cnc+ss</i>	two sets of an buds and two lr; one S	2I/I'
E10	4	e	<i>six3</i>	smallest amount of shared anterior tissue	2J/J'
E21	6	pre-an	<i>ss</i>	(putative) later stage ADE as shown in E09	S2A/A'/B
E22	6.1	(unclear)	---	(putative) later stage ADE as shown in E09	S2C/D
E23	1.2	e	<i>Pax6+en</i>	younger stage ADE as shown in E10; smallest amount of shared anterior tissue	S2E/E'/F
Class-II: Cases of <i>Duplicitas posterior</i> with asymmetrically duplicated posterior germ bands					
E11	4	(unclear)	<i>ss</i>	minor germ band dorso-ventrally compressed; affiliation of segments unclear in minor germ band	3A/A'
E12	4	dito	<i>ss+Antp</i>	dito	3B/B'
E13	4	dito	<i>ss+Antp</i>	dito	3C/C'
E14	6	dito	---	minor germ band “sits” on dorsal side of major germ band	3D/D'

E24	6	dito	---	minor germ band dorso-ventrally compressed; affiliation of segments unclear in minor germ band	S2G
E25	6	dito	---	dito	S2H/I
Class-III: Cases of <i>Duplicitas posterior</i> - separate germ bands without anterior head segments					
E15	4	---	<i>cnc+ss</i>	most anterior identified segment in both germ bands: md; most anterior tissue of unclear affiliation	4A/A'-C/C'
E16	4.1	---	<i>Antp</i>	most anterior identified segment in both germ bands: pmx; most anterior tissue of unclear affiliation	4D/D'-F/F'
Class-IV: Cases of <i>Duplicitas posterior</i> - fused germ bands without anterior head segments					
E17	5	(unclear)	---	most anterior identified segment: md; most anterior tissue of unclear affiliation	5A/B
E18	6	dito	<i>cnc+ss</i>	dito	5C-E
E19	6	dito	<i>cnc+ss</i>	dito	5F-H
E20	6	dito	---	dito	5I-J

Supplementary Material

[Click here to download Supplementary Material: Fig.S1_Gm-ss and Gm-six3.tif](#)

Supplementary Material

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Supplementary Material

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