Research

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Regressive evolution of the arthropod tritocerebral segment linked to functional divergence of the Hox gene *labial*

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The intercalary segment is a limbless version of the tritocerebral segment and is present in the head of all insects, whereas other extant arthropods have retained limbs on their tritocerebral segment (e.g. the pedipalp limbs in spiders). The evolutionary origin of limb loss on the intercalary segment has puzzled zoologists for over a century. Here we show that an intercalary segment-like phenotype can be created in spiders by interfering with the function of the Hox gene *labial*. This links the origin of the intercalary segment to a functional change in *labial*. We show that in the spider *Parasteatoda tepidariorum* the *labial* gene has two functions: one function in head tissue maintenance that is conserved between spiders and insects, and a second function in pedipalp limb promotion and specification, which is only present in spiders. These results imply that *labial* was originally crucial for limb formation on the tritocerebral segment, but that it has lost this particular subfunction in the insect ancestor, resulting in limb loss on the intercalary segment. Such loss of a subfunction is a way to avoid adverse pleiotropic effects normally associated with mutations in developmental genes, and may thus be a common mechanism to accelerate regressive evolution.

1. Introduction

The evolution of novel animal body plans requires the evolution of novel traits. Often, however, new bodyplans also emerge by the loss of features and organs. Examples of this so-called regressive evolution include eye loss in cavefish [1], wing loss in ants [2] or the loss of limbs in the snake bodyplan [3]. Another textbook example for regressive evolution is limb loss on the intercalary segment in insects [4]. The limbless intercalary segment is an integral component of the head capsule of all insects and, therefore, must be phylogenetically older than all extant insect groups. The controversy about its origin and the genetic basis for its limblessness ranks among the longest standing unsolved problems in zoology.

Anatomically, the intercalary segment corresponds to the tritocerebral segment in other arthropods [5]. In contrast to insects, several non-insect arthropods (e.g. spiders) have retained the limbs of the tritocerebral segment, thus providing a valuable model for tritocerebral segment development before the onset of regressive evolution in the insect ancestor. We have, therefore, studied the developmental genetic mechanisms of tritocerebral segment formation in the spider *Parasteatoda tepidariorum* (previously placed in the genus *Achaearanea* [6]). In spiders, the tritocerebral segment bears the pair of pedipalps [5], a multi-functional appendage type used in feeding, sensory perception and for many specialized purposes like visual communication, stridulation or sperm transfer (reviewed in [7]). The developmental genetic mechanisms needed to form the pedipalpal segment and its appendages are, however, not known.

One gene that is strongly expressed in the tritocerebral segment of all arthropods studied so far is the Hox gene *labial* (*lab*) [8]. On the one hand, this strong
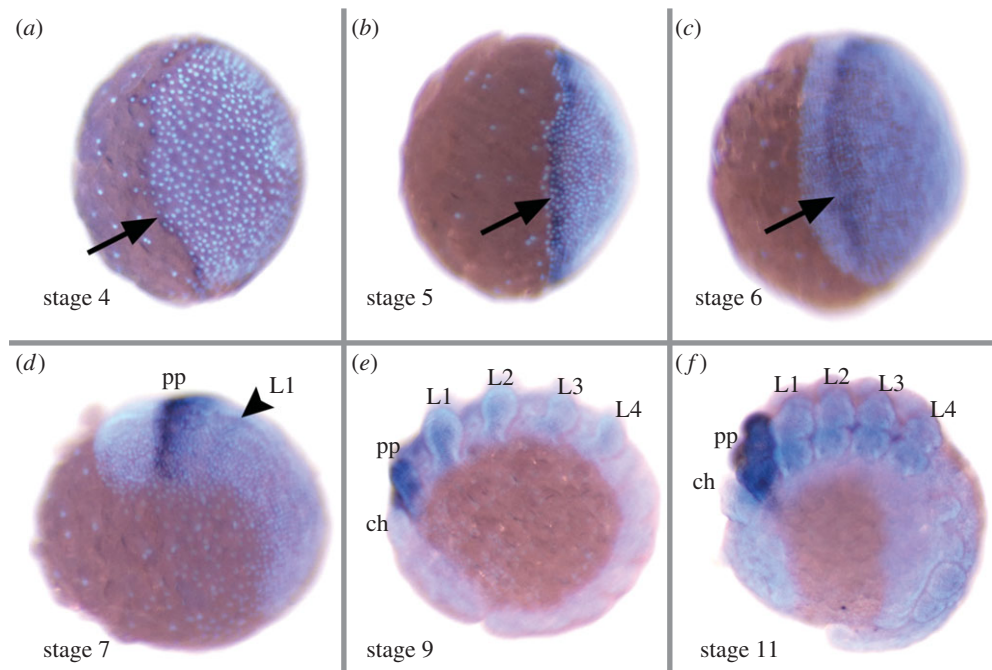
Figure 1. Developmental expression of *lab-1*. (*a*–*c*) Lateral view of the germ disc at stage 4 (*a*), stage 5 (*b*) and stage 6 (*c*). (*d*–*f*) Lateral view of germ band stages, anterior to the left, at stage 7 (*d*), stage 9 (*e*) and stage 11 (*f*). Arrows indicate expression in the presumptive pedipalp segment tissue. The arrowhead indicates beginning weak expression in the L1 segment. Abbreviations: ch, chelicera; L1–L4, walking legs 1–4; pp, pedipalp. (Online version in colour.)

evolutionary conservation indicates an important role of *lab* in the arthropod tritocerebral segment. On the other hand, *lab* expression is conserved in all arthropods, regardless of the presence of limbs on the tritocerebral segment, and therefore, a possible role of *lab* in the control of limblessness has been dismissed previously [9]. In this work, we have investigated the role of *lab* in the spider *P. tepidariorum* and its importance for limb formation in the spider tritocerebral segment, i.e. the pedipalpal segment. We show that apart from a role in head tissue maintenance already known from insects, spider *lab* has a role in appendage initiation and development that is not present in insects. We propose that the loss of this function in the insect ancestor initiated regressive evolution in the insect tritocerebral segment and thus gave rise to the limbless intercalary segment.

2. Material and methods

(a) Spider colony and gene cloning

Embryos and adults of *P. tepidariorum* were obtained from our Göttingen strain of *P. tepidariorum* for all experiments. The animals were kept separately in small plastic vials at 25°C and controlled light cycle (12 L : 12 D cycle). Isolation of gene fragments from *P. tepidariorum* was performed according to standard molecular cloning techniques. Sequences of primers and accession numbers are included in the electronic supplementary material.

(b) RNA interference

Parental RNA interference (RNAi) was performed as described [10] with minor modifications. To exclude off-target effects non-overlapping fragments were injected separately (electronic supplementary material, figure S4). Full documentation of the injections is also available in the electronic supplementary material.

(c) *In situ* hybridization, DNA labelling and imaging

In situ hybridization and nuclear staining with Sytox Green were performed as described [11,12] with minor modifications (details

are included in the electronic supplementary material). TUNEL detection of fragmented DNA was done as described previously [13]. Images were captured with a Zeiss Axioplan-2 microscope or with a Leica dissection microscope equipped with an Intas digital camera and UV light. Confocal z-stacks of larvae were captured by using a Zeiss LSM 510 microscope.

3. Results

(a) Expression of *labial* in *Parasteatoda tepidariorum*

P. tepidariorum has two paralogous copies of the *lab* gene (see electronic supplementary material for sequence accession numbers). One of these genes, that we denote as *lab-1*, is expressed in the early embryo (starting at stage 4; staging after [14]) in a circumferential ring along the rim of the germ disc (figure 1*a*), which becomes broader during stage 5 (figure 1*b*).

During the transformation of the germ disc into the bilaterally symmetric germ band, which starts at stage 6, the cephalic region as well as the cheliceral and the pedipalpal segments are patterned via a dynamic anterior to posterior wave of gene expression. This dynamic wave is mainly organized by an autoregulatory loop that involves Hedgehog-signalling and the transcription factors *orthodenticle* and *odd-paired* [15,16]. The expression domains of several genes that are initially located at the rim of the germ disc during stage 5, are travelling to a more posterior position during stages 6–8. Because *lab-1* is co-expressed with several of these 'travelling genes' during stage 5, the expression ring of *lab-1* is also subject to this wave of gene expression and is relocated from the rim towards the presumptive pedipalpal segment (figure 1*c*). After the formation of the germ band at stage 7, *lab-1* is strongly expressed in the pedipalpal segment and weaker expression is also detected in the first walking leg segment (figure 1*d*). In later developmental stages (starting with stage 9) there is also very faint expression in the remaining walking leg segments (figure 1*e,f*). The second *lab* gene, that we denote as *lab-2*, is
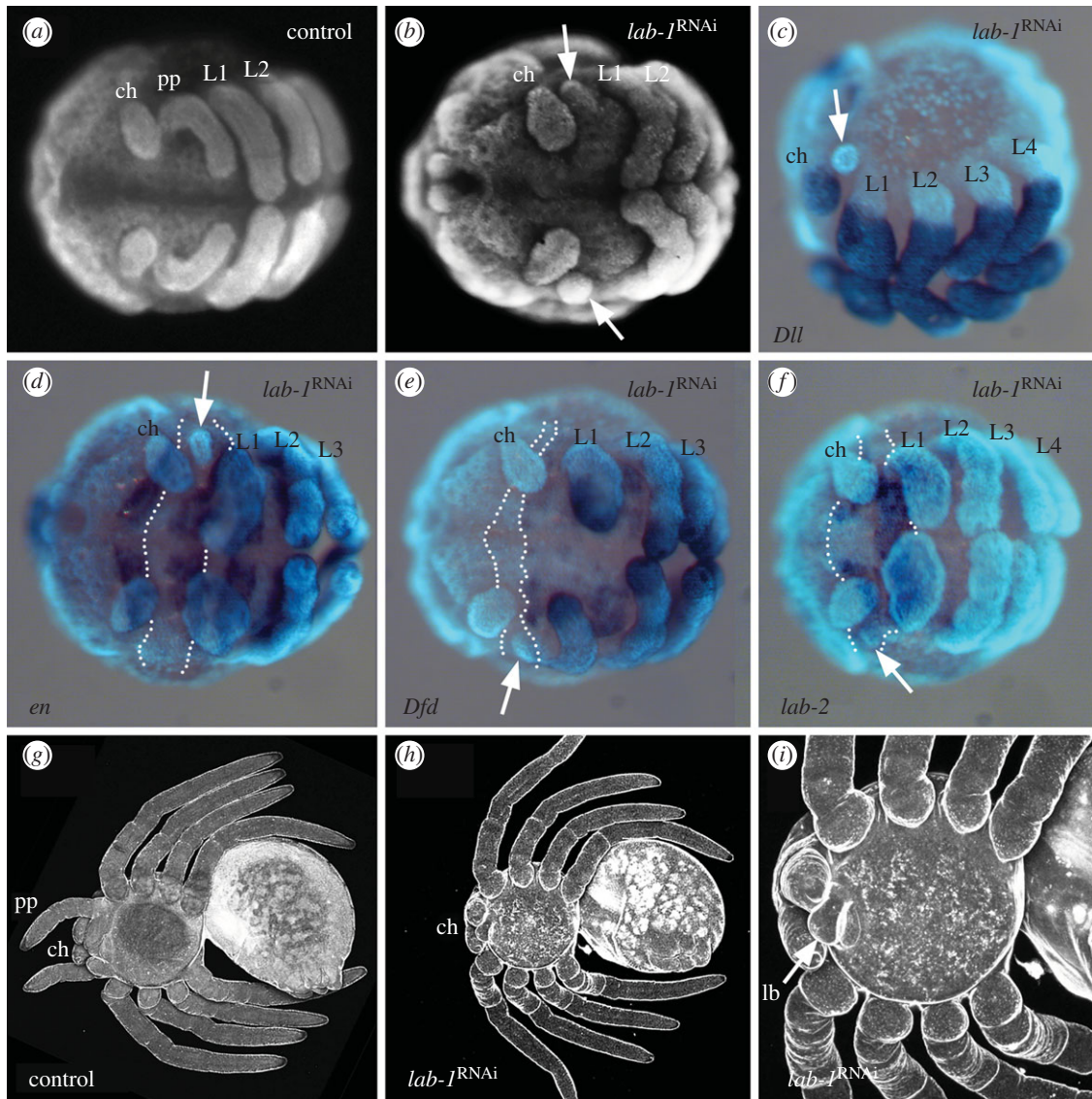
Figure 2. Phenotypes after *lab-1* RNAi. (*a,b*) Epifluorescent images stained with Sytox Green. (*a*) Wild-type embryo. (*b*) *lab-1* RNAi embryo. (*c–f*) Combined epifluorescent images (light blue) and light microscopy images of *lab-1* RNAi embryos stained for expression of *Dll* (*c*), *en* (*d*), *Dfd* (*e*) and *lab-2* (*f*). The arrows in (*b–f*) point to vestiges of the pedipalp. The dotted lines in (*d–f*) indicate the approximate area of the pedipalp segment. (*g–i*) Confocal images of larvae. (*g*) Wild-type larva. (*h*) *lab-1* RNAi larva. (*i*) Magnified view of prosoma of a *lab-1* RNAi larva, showing the labium (lb). All panels in ventral view and anterior up. See figure 1 for abbreviations. (Online version in colour.)

transcribed only late and no expression is detected before stage 6. RNAi with *lab-2* did not result in detectable phenotypes.

(b) *Pt-lab-1* RNAi can create an intercalary segment-like phenotype in the spider

RNAi with *lab-1* function led to characteristic phenotypes. The *lab-1* RNAi phenotypes comprise defects in the pedipalpal and first walking leg segment, thus corresponding well with the early expression domains of *lab-1* in the pedipalpal and first walking leg segments. There are two major categories of phenotypes: in category 1 (denoted in purple in electronic supplementary material, figure S1), only the pedipalpal segment is affected. In category 2 (denoted in green and orange in electronic supplementary material, figure S1), there are additional defects of different severity in the L1 segment. The pedipalp appendages are mostly completely reduced, but small remnants may be present (figure 2*b*). The expression of the appendage marker gene *Distal-less* (*Dll*) is entirely absent from the pedipalpal segment in *lab-1*

RNAi animals (figure 2*c*; electronic supplementary material, figure S2), indicating that the specification of appendages in this segment is disrupted. In category 1 and weakly affected category 2 animals the pedipalpal segment itself is reduced in size, but is otherwise morphologically normal (except for the missing appendages). This is also confirmed by genetic marker expression. The segmental borders are specified normally as illustrated by the expression of the segment polarity marker *engrailed* (*en*) at the posterior boundary of the limbless pedipalp segment (figure 2*d*). The segment also retains its normal identity as indicated by the expression of other Hox genes. In the wild-type, *lab-2* and *Deformed* (*Dfd*) are expressed in the pedipalpal and in the walking leg segments, respectively, and these patterns are unchanged in *lab-1* RNAi animals (figure 2*e,f*). These results thus demonstrate a role of *lab-1* during pedipalp appendage specification.

In category 2 animals, there are additional defects in the L1 segment. In weaker phenotypes, in the L1 segment the legs are only shortened and broadened. In stronger phenotypes, the legs in L1 are entirely missing (electronic supplementary

material, figure S3a). Initially, in these embryos the pedipalpal and the L1 segment are physically present (at least partially) as indicated by the gap between the cheliceral segment and the L2 segment (electronic supplementary material, figure S3b). But the absence of segmental gene expression (e.g. stripes of *en*) in this area points to severe developmental deficiencies, and the rate of cell death is strongly increased in the entire prosomal part of the germ band (electronic supplementary material, figure S3c,d), which is also observed after *lab* loss in insects [17–19]. This suggests that *lab-1* is also generally required for tissue maintenance in the entire anterior portion of the germ band, including the pedipalpal and L1 segments.

Only few *lab-1* RNAi embryos survive and reach the larval stage, and no animals survive beyond the first larval instar. These larvae completely lack the pedipalpal appendages (figure 2g,h), but other elements of the pedipalpal segment are present, demonstrated by the presence of the labium (figure 2i), which is a protrusion of the sternum (the ventral armour plate of the spider exoskeleton) and is the modified sternite of the pedipalpal segment [20]. Thus, the morphology of the pedipalpal segment in these *lab-1* RNAi larvae strikingly resembles the intercalary segment in insects. We did not observe homeotic transformations in *lab-1* RNAi animals, neither in the first larval instar nor in the embryo. The transformation of dorsal head tissue towards thoracic fate has been described for *Drosophila melanogaster*, but only in the adult, and not in the embryo or larva [17]. Thus, the available data from *P. tepidariorum* are compatible with the data from *D. melanogaster*, because in both cases no transformations are present before the adult stage. The late role in homeosis might also be present in *P. tepidariorum*, but because *lab-1* RNAi animals die before reaching the adult stage, we were not able to investigate this possibility.

(c) *Pt-lab-1* can transform the L1 segment towards pedipalp identity

Because *lab-1* has a role in pedipalp appendage specification, we also asked whether *lab-1* can play this role in other segments, i.e. is ectopic expression of *lab-1* able to initiate pedipalp appendage development in other segments? Because targeted misexpression is not yet available in *P. tepidariorum*, we achieved activation of *lab-1* in the L1 segment by interfering with the function of the Hox gene *Dfd*. *Dfd* is expressed in all four walking leg segments and thus posteriorly adjacent to the pedipalpal segment [21]. RNAi with *Dfd* leads to the ectopic expression of *lab-1* (figure 3d,e), but not of *lab-2* (figure 3f), in the L1 segment. As a consequence, the L1 legs are transformed towards pedipalp identity. Pedipalps can easily be distinguished from legs: they differ from the legs by being shorter and missing one limb segment (the metatarsus). The pedipalps are also characterized by the presence of a ventral outgrowth, the gnathendite, that in the embryo is further identified by its separate expression of *Dll*. In addition, late embryonic stages possess an egg tooth on the outside of the basal portion of the pedipalps, which is used for penetration of the eggshell. In *Dfd* RNAi animals the transformation of L1 into pedipalp is either partial or complete (summary in electronic supplementary material, figure S1). The partially transformed L1 leg is shortened and proximally thickened. In complete transformations, the transformed L1 leg is morphologically indistinguishable from the normal pedipalp (figure 3a,b). In the embryo, the gnathendite of the transformed L1 leg expresses

Dll and the egg tooth is also present (figure 3g,g′). In nymphs that have completed cuticle differentiation and limb joint formation, it can be demonstrated that the fully transformed L1 leg indeed consists of only six limb segments and is lacking the metatarsus (figure 3c). The fact that only *lab-1*, but not *lab-2* is misexpressed in the L1 segment after *Dfd* RNAi, indicates that the specification of pedipalp appendage identity observed in the transformed L1 legs is the specific effect of *lab-1*.

4. Discussion

The Hox gene *labial* shows a highly conserved expression pattern in the tritocerebral segment in all arthropods [8] and even in onychophorans [22,23], most probably the arthropod sister group [24,25]. Consistent with this observation, our results identify a highly conserved role of *labial* in head tissue maintenance that has been known already from functional studies in *D. melanogaster* and the beetle *Tribolium castaneum*. In *D. melanogaster* loss of *lab* function leads to increased cell death in the head region and eventually to the loss of several head segments including the intercalary, mandibular, maxillary and labial segments, hence the gene name [17]. A similar effect has also been observed in the beetle *T. castaneum*, where *lab* RNAi phenocopies show increased cell death in the head including at least the intercalary, mandibular and maxillary segment [18].

However, our results also reveal a second role of *lab-1* in the specification and formation of the pedipalp limbs. This role is apparently absent from insect *lab* homologues, because they lack the corresponding limbs altogether, although *lab* is strongly expressed in the insect intercalary segment. Intriguingly, the loss of this *lab-1* function in spiders removes the genetic cues to form appendages on the pedipalpal segment and thus experimentally recapitulates the origin of limb loss on the tritocerebral segment. The result is a pedipalpal segment phenotype that is strikingly similar to the insect intercalary segment, not only in terms of morphology but also in terms of gene expression. We therefore suggest that the dual role seen in *lab-1* in *P. tepidariorum* represents the ancestral condition of the *labial* gene in arthropods, and is retained in most chelicerates and probably also in the second antennal segment of crustaceans [26]. The role in head tissue maintenance appears to be indispensable and is highly conserved, but the role in limb formation has been lost in insects. Interestingly, insects are not the only arthropods that lack limbs on the intercalary segment homologue. Myriapods also lack limbs on the tritocerebral segment [27,28]. This limb loss has previously been regarded as homologous in myriapods and insects and served as the key character for uniting the two groups in the taxon Antennata [28], but recent molecular phylogenies do not support the Antennata taxon and place the myriapods at the base of all mandibulate arthropods [29]. Accordingly, limb loss on the tritocerebral segment in insects and in myriapods has evolved by convergence. Another intriguing case is the Pycnogonida (sea spiders), an enigmatic arthropod group usually considered as relatives of spiders and other chelicerates [29,30]. Some species of Pycnogonida lack the pedipalp limbs, while other species have them fully formed [31]. Thus, in pycnognids limb loss on the tritocerebral segment apparently evolved several times within the group. The role of *lab* in myriapods and pycnogonids, however, is not yet known and it will therefore be interesting to study, whether their limblessness is also linked to evolutionary changes in the role of *lab*.
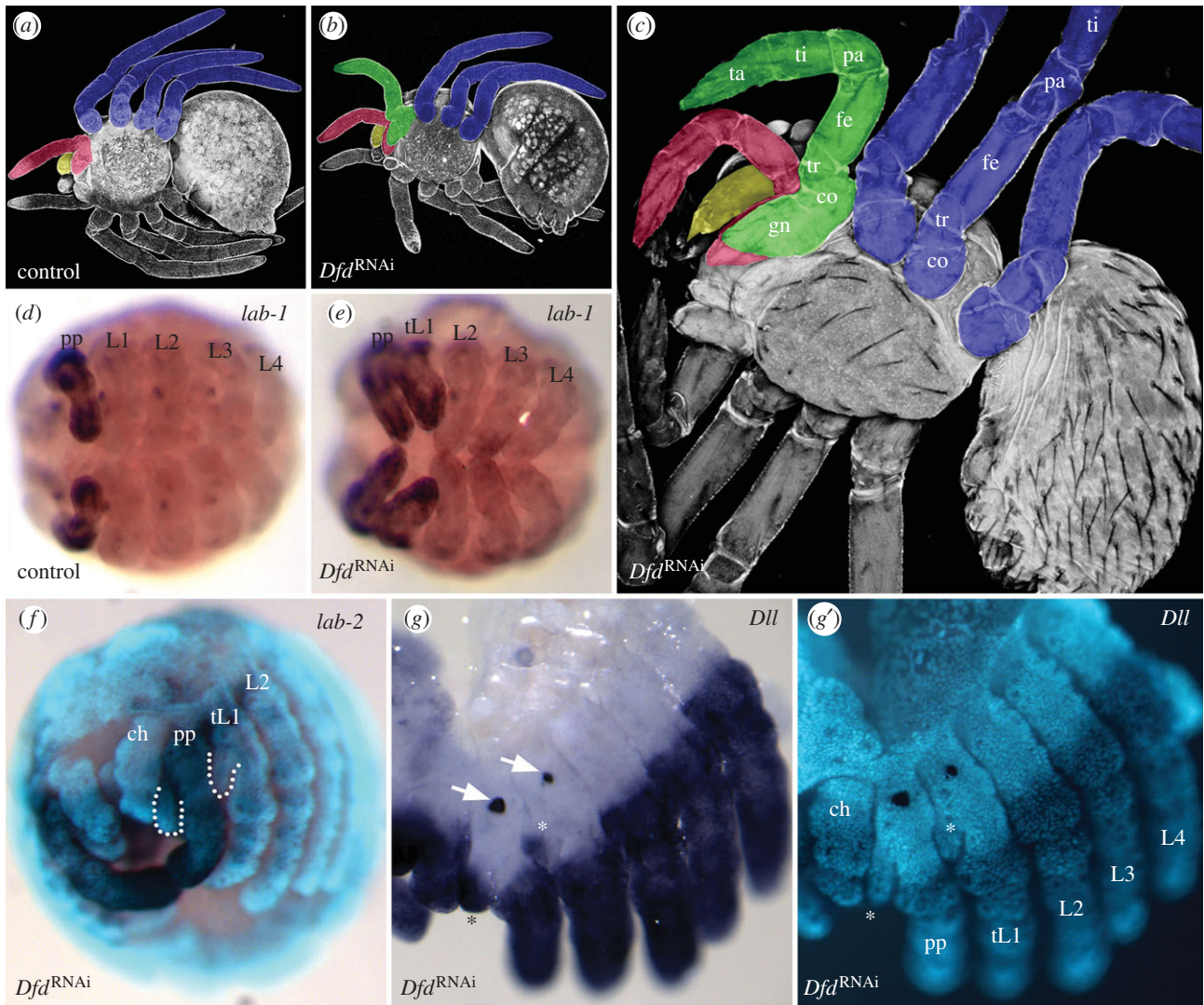
Figure 3. Phenotypes after *Dfd* RNAi. (*a*–*c*) Confocal images, ventral view. Appendages on the right body half have been colourized to indicate their identity (blue, leg; red, pedipalp; yellow, chelicera; green, first leg transformed into pedipalp identity). Limb segments are first visible in the nymph (indicated in *c*). (*a*) Control larva. (*b*) *Dfd* RNAi larva. (*c*) *Dfd* RNAi first nymphal instar. (*d* and *e*) Light microscopical images of stage 11 embryos stained for *lab-1*, ventral view. (*d*) Control embryo. (*e*) *Dfd* RNAi embryo (tL1, transformed L1). (*f*) *lab-2* expression in *Dfd* RNAi embryo. Combined epifluorescent and light microscopy image. Dotted lines denote gnathendites in pedipalp and tL1. (*g*,*g*′) Expression of *Dll* in a *Dfd* RNAi embryo. Light microscopy image (*g*) and corresponding epifluorescent image (*g*′). Arrows and asterisks point to the egg tooth and *Dll* expressing gnathendite, respectively, in the pedipalp and the tL1. co, coxa; fe, femur; gn, gnathendite; pa, patella; ta, tarsus; ti, tibia; tr, trochanter. See figure 1 for other abbreviations. (Online version in colour.)

The role of selection in the regressive evolution of organs was already discussed by Charles Darwin in 'On the origin of species' [32]. Using the loss of eyes in cavefish as an example, he realized that the loss of eyes is neither particularly advantageous, nor would the preservation of eyes be a major disadvantage. It appeared to Darwin that cavefish have lost their eyes simply because it does not matter whether they have eyes or not. Owing to this line of reasoning, it is generally assumed that regressive evolution is caused by neutral evolution and genetic drift, rather than natural selection. However, recent studies provide evidence for strong positive selection for the reduction of traits [33,34], especially if the reduced trait is genetically coupled to another highly adaptive trait (antagonistic pleiotropy) [35,36]. Although limb loss on the tritocerebral segment has apparently evolved several times, the selectional benefit of a limbless tritocerebral segment is unclear at the moment. At any rate, losing the function of a gene is especially difficult for developmental genes, because they usually have more than one function and their loss would lead to multiple defects with negative rather than positive or neutral effect. Our results with *lab-1* suggest that the loss of only a particular subfunction of a developmental gene is one way to avoid adverse pleiotropic effects, and may thus be a common mechanism to facilitate regressive evolution in animals.

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