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# **COMPARATIVE FUNCTIONAL ANALYSES OF ULTRABITHORAX REVEAL MULTIPLE STEPS AND PATHS TO DIVERSIFICATION OF LEGS IN THE ADAPTIVE RADIATION OF SEMI-AQUATIC INSECTS**

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Invasion of new ecological habitats is often associated with lineage diversification, yet the genetic changes underlying invasions and radiations are poorly understood. Over 200 million years ago, the semi-aquatic insects invaded water surface from a common terrestrial ancestor and diversified to exploit a wide array of niches. Here, we uncover the changes in regulation and function of the gene *Ultrabithorax* associated with both the invasion of water surface and the subsequent diversification of the group. In the common ancestor of the semi-aquatic insects, a novel deployment of Ubx protein in the mid-legs increased their length, thereby enhancing their role in water surface walking. In derived lineages that specialize in rowing on the open water, additional changes in the timing of *Ubx* expression further elongated the mid-legs thereby facilitating their function as oars. In addition, Ubx protein function was selectively reversed to shorten specific rear-leg segments, thereby enabling their function as rudders. These changes in *Ubx* have generated distinct niche-specialized morphologies that account for the remarkable diversification of the semi-aquatic insects. Therefore, changes in the regulation and function of a key developmental gene may facilitate both the morphological change necessary to transition to novel habitats and fuel subsequent morphological diversification.

**KEY WORDS:** Adaptation, adaptive radiation, allometry, development, macroevolution, variation.

Invasion and radiation into new habitats may often require the evolution of novel morphologies to meet the challenges and exploit the opportunities of new life styles (Liem 1973; Schluter 2000; Losos 2009). Evolutionary developmental biology has made great progress toward understanding the genetic mechanisms underlying the evolution of numerous morphological traits (Averof and Patel 1997; Abzhanov et al. 2004; Moczek and Rose 2009; Chan et al. 2010; Reed et al. 2011; Jones et al. 2012; Khila et al. 2012; Rohner et al. 2013; Konopova and Akam 2014). However, how these mechanisms are associated with radiations remains poorly understood. Here, we focus primarily on the infraorder Gerromorpha, a monophyletic group of semi-aquatic insects thought to have arisen in the Triassic 250–200 million years ago (Damgaard 2008). Descended from fully terrestrial ancestors, Gerromorphans invaded the water surface and radiated into remarkable new forms to take advantage of these new niches, and now include some 2000 species (Andersen 1979, 1982; Hebsgaard et al. 2004; Damgaard et al. 2005; Damgaard 2008a) (Fig. 1).



**Figure 1.** Phylogenetic relationships and diversity in relative leg length in the semi-aquatic insects and outgroups. Highlighted in blue is the under water swimmer *Notonecta*, which has independently transited to life under water. In black is the ground walking *Oncopeltus*, which is exclusively terrestrial and used here as a terrestrial outgroup. In green are the mesoveliid *Mesovelia* and the veliid *Microvelia*, both are water surface walking semi-aquatic insects. In red are the three-water surface rowing gerrids *Metrobates*, *Gerris*, and *Limnoporus*. Small red boxes represent the evolution of surface rowing as a novel mode of locomotion, and the characteristics derived leg length plan, which is though to have evolved multiple times in the Viliidae and once in the common ancestor of the Gerridae.

While the sister taxon Nepomorpha transited to life under water (Fig. 1), extant Gerromorpha make their living on wet surfaces, at the terrestrial-aquatic boundary, in the near shore and on open water surfaces, including marshes, lakes, ponds, streams, and even oceans (Andersen 1982). The move to the water surface required new means of locomotion (walking and rowing on the water surface), which in turn required evolutionary change in the appendages and associated musculature (Andersen 1976). There is a great diversity in relative leg lengths, both ratios of fore- to mid- to rear-legs, and in the ratios of femur to tibia to tarsus. Perhaps the most substantive evolutionary change to the legs is the transition from the ancestral "rear-legs longer than mid-legs" (indicated by blue, black, and green boxes in Fig. 1) to the derived "mid-legs longer than rear-legs" leg length plan (indicated by red boxes in Fig. 1). The Mesoveliidae, the most basally branching group, are water walkers (at the near shore and land-water interface) and the Veliidae, a paraphyletic group, include both water walkers and rowers (on the open water). Within the Veliidae, the water walkers have the ancestral (rear-legs longer than mid-legs) appendage plan, and the rowers have the derived (mid-legs longer than rear-legs) leg length plan (Andersen 1982; Damgaard et al. 2005). The derived leg length plan and rowing on the open water surface is a general feature of the Gerridae (Andersen 1982; Damgaard et al. 2005). The derived leg length plan is generally agreed to have evolved multiple times independently: multiple times in the Veliidae and once in the ancestor of Gerridae (Andersen 1979, 1982; Hebsgaard et al. 2004; Damgaard et al. 2005; Damgaard 2008a).

The Hox gene *Ubx* is known to establish key morphological differences among thoracic segments (Akam 1998a; Stern 1998;

Ronshaugen et al. 2002; Pavlopoulos et al. 2009; Konopova and Akam 2014), and to regulate the length of locomotory appendages in many insects including the Gerridae (Stern 2003; Mahfooz et al. 2007; Khila et al. 2009). In exclusively terrestrial insects, rear-legs tend to be longer than the mid-legs. Such is the case of the distantly related orthopteran Acheta domesticus and the closely related heteroperan Oncopeltus fasciatus. In these insects, Ubx protein expression is anteriorly restricted to embryonic third thoracic segment (T3) and functions to lengthen the rear-legs that this segment bears, relative to the mid-legs on T2 where Ubx is not expressed (Mahfooz et al. 2004, 2007). In the water strider Gerris buenoi the mid-legs, which function as propelling oars, are longer than the rear-legs, which function as steering rudders (Andersen 1976; Hu et al. 2003). In contrast to Acheta and Oncopeltus, we have previously demonstrated that in G. buenoi, Ubx expression is now extended anteriorly to T2 (Khila et al. 2009), where its effect is to elongate the mid-legs, yet shorten the rear-legs, thereby establishing the novel leg length plan (Khila et al. 2009). Across the semi-aquatic insects, either walkers or rowers, there exists substantial variation in relative segment length within individual legs (contrast the rear femurs in Metrobates and Gerris, Fig. 1). Thus, both major changes in total leg lengths, and minor changes in leg segment lengths are associated with diversification in the group.

Here, we conduct a broad comparative analysis within a phylogenetic framework to trace the developmental, evolutionary, and ecological origins of appendage adaptive diversification (Fig. 1). To this end, we analyzed Ubx expression using immunohistochemistry and function using RNA interference across seven representative species within four families including outgroups. Using this integrative approach, we uncovered two major evolutionary steps in Ubx function and regulation that are phylogenetically associated with the ecological transitions of semiaquatic insects to the water–land interface and to the open water surface. In addition, smaller scale evolutionary changes in Ubx expression and function appear to be associated with the adaptive diversification within these habitat zones.

# Methods species sampling

All species used in this study were collected in the vicinity of Montreal, Quebec, Canada. *Notonecta undulata* were collected in Beaver Lake Park in Montreal, *Mesovelia mulsanti* and *Limnoporus dissortis* in "Rivière l'Acadie" east of Montreal, *Rhagovlia obesa, Metrobates hesperius*, and *Microvelia americana* in "Rivière du Nord" north of Montreal. All species were reared in water tanks at 25°C and fed with live crickets and frozen flies. Females were provided with floating pieces of styrofoam or plastic plants to oviposit their eggs, which were then employed in subsequent developmental experiments.

#### UBX CLONING

Total RNA was extracted from developing embryos of each species and used as a template in a first strand complementary DNA synthesis reaction (Invitrogen). The cDNA was then used as a template to conduct PCR using *Ubx* forward and reverse primers (listed in Table S1). In the case of *N. undulata*, the total RNA was used to synthesize a 3' Rapid Amplification of cDNA Ends (RACE) reaction (Invitrogen) to obtain a 3'RACE template. The cDNA or 3' RACE cDNA were used as a template to clone an *Ubx* fragment from each species using PCR. Primer combinations as well as the GenBank accession numbers of the *Ubx* sequences obtained in each species are listed in Table S1.

#### **EMBRYO DISSECTION AND FIXATION**

Embryos were treated with 25% bleach for 2–3 minutes to weaken the chorion. Embryos were then dissected with fine forceps in PTW (1 X PBS buffer and 0.05% Tween-20), followed by a 15minute fixation in 4% formaldehyde in PTW. Embryos of all species were treated similarly and all steps were conducted at room temperature unless otherwise indicated.

# Specificity and Staining with the Fp6.87 Anti-Ubda Antibody

The FP6.87 anti-UbdA antibody (courtesy of Rob White) recognizes both Ubx and Abdominal-A (Abd-A) proteins across insects (White and Wilcox 1984; Kelsh et al. 1994; Mahfooz et al. 2004; Khila et al. 2009). The domain of expression of each of these two proteins is well established across insects (White and Wilcox 1984; Kelsh et al. 1994; Mahfooz et al. 2004; Khila et al. 2009). In the water strider *Gerris buenoi*, Ubx is expressed in the second and third thoracic segments as well as the first abdominal segment (Khila et al. 2009). The AbdA domain, however, expands from the second abdominal segment to the eighth abdominal segment (Khila et al. 2009). In *G. buenoi* embryos treated with *Ubx* RNAi, only Abd-A protein is detected by UbdA antibody in the abdominal segments (Khila et al. 2009). Therefore, the FP6.87 anti-UbdA antibody is a highly reliable reagent to reveal the domain of Ubx expression in the thoracic segments of all species studied.

#### STAINING

After fixation, embryos are washed several times in PTW, and then incubated twice 20 minutes in PBT (1X PBS and 0.3% Triton-X 100). A blocking step follows where embryos are incubated 1 hour in PAT (1X PBS, 0.1% Triton-X 100, and 10% Normal Goat Serum). Blocked embryos are then incubated with the FP6.87 antibody at 1/5 dilution in blocking solution overnight at 4°C. Embryos are washed five times, for 10 minutes each time, in blocking solution, then incubated with an anti-mouse secondary antibody conjugated to horseradish peroxydase for two hours at room temperature. Embryos are washed four times in PBT and two times in PTW, 10 minutes each. Finally, expression was revealed in a reaction solution containing DAB, NiCl<sub>2</sub>, and 0.002% H2O2. Reaction was stopped using several washes in PBT, embryos were then washed in PBS-Glycerol, mounted on slides and observed in a Zeiss Axioimager microscope.

#### **UBX** RNAI

For each species, parental Ubx RNAi was performed by injecting a solution of Ubx double stranded RNA (ds-Ubx) into adult females. Phenotypes were then scored in the embryonic progeny of the injected females. ds-Ubx of all species, except Notonecta, was synthesized based on N-terminal sequences that exclude the homeodomain to avoid cross-targeting of other homeotic proteins (Fig. S1). Notonecta ds-Ubx was synthesized based on Ubx 3' UTR sequence, which also excludes the homeodomain, using a Notonecta Ubx-specific forward primer and reverse primer designed based on the 3' RACE universal adaptor (Invitrogen, Fig. S1). Forward and reverse primers both containing the T7 RNA polymerase promoter (see Table S2 for primer sequences) were synthesized and used to amplify an in vitro transcription template that is flanked by T7 promoters. The portions of the Ubx sequences used to synthesize ds-Ubx in each species are highlighted in Figure S1. In vitro transcription, using T7 RNA polymerase, generates both sense and anti-sense transcripts, which are automatically annealed into double stranded RNA (dsRNA) while the reaction progresses at 37°C without any further treatment.



**Figure 2.** Ubx expression and function in the aquatic Nepomorphan Notonecta and the most basally branching Gerromorphan Mesovelia. (A and G) Early Ubx expression in *Notonecta* (A) and *Mesovelia* (G) embryos showing that in both species Ubx is expressed in the rear-leg at this early stage. (B and H) Late expression showing that Ubx persists in the rear-leg and is absent in the mid-leg in *Notonecta* embryos (B), but appears in both legs in *Mesovelia* embryos. Black arrows indicate legs expressing Ubx. (C and I) Leg arrangement of late embryos of *Notonecta* (C) and *Mesovelia* (I). Open arrowheads indicate position of mid-leg tips and black arrowheads indicate rear-leg tips. (D and J) Effect of *Ubx* RNAi treatment on leg length in late embryos of *Notonecta* (D) and *Mesovelia* (I) as indicated by positions of the tips of mid- and rear-legs. (E and K) hatched nymphs of *Notonecta* (E) and *Mesovelia* (K) showing that relative leg length is established during embryogenesis. (F and L) hatched nymphs showing the effect of *Ubx* RNAi on relative leg length in *Notonecta* (F) and *Mesovelia* (L).

ds-*Ubx* is purified using Qiagen RNeasy purification kit and eluted in Spradling injection buffer (Spradling and Rubin 1982). Adult females were anaesthetized using carbon dioxide, immobilized on double sticky tape and injected with ds-*Ubx* or ds-yfp (as negative control) at 1 mg/ml concentration. Injected females were replaced in water tanks and fed daily. Embryos were allowed to develop at room temperature and screened for leg length and other phenotypes characteristic to *Ubx* RNAi, such as ectopic legs on the first abdominal segment. The number of injected females per species, and the number of embryos either normal or showing *Ubx* phenotypes can be retrieved in Table S3. The RNAi penetrance (percentage of the progeny with *Ubx* phenotype) varies between 45% (*Metrobates*) and 100% (*Mesovelia*) (Table S3). *Metrobates* proved to be sensitive to injection as female mortality was high and embryo output was low in both *Ubx*- and control-injected females.

#### LEG MEASUREMENTS AND STATISTICAL ANALYSES

To measure the effect of *Ubx* RNAi on leg length, we selected a sample of 10 embryos (N = 10 in Fig. 5) where *Ubx* phenotype is unambiguously recognizable by the formation of an ectopic leg on the first abdominal segment that is a hallmark of *Ubx* RNAi phenotype across insects (Angelini et al. 2005; Mahfooz et al.

2007; Khila et al. 2009) (Figs. 2, 3, and 4), for each species and treatment group, except in *Mesovelia* where N = 7. These embryos were chosen at a late stage of embryogenesis (same stage as in Fig. 2C–D and I–J; Fig. 3D–E; Fig. 4C–D, I–J, and O–P) to standardize the analysis across all species. For *Metrobates*, injection led to high female mortality and low progeny numbers for both *Ubx* and control double-stranded RNA. We therefore did not perform statistical analyses on this species. A sample of WT and *Ubx* RNAi embryos were first individually measured to collect body length data and then dissected to recover legs. Legs were mounted in Hoyers in slides and incubated at 60°C in an oven for two hours. Leg measurements were performed using a Zeiss dissecting scope and Axiovision software. Data for each leg segment in each species were analyzed using ANCOVA, with two treatments (WT and *Ubx* RNAi) and embryo length as the covariate.

#### SCANNING ELECTRON MICROSCOPY

Control and *Ubx* RNAi hatched first instar nymphs of *Mesovelia* were washed in absolute ethanol, transferred to acetone and subjected to critical drying. These samples were then coated using a Baltec MED 020 carbon coater. Scanning Electron Micrographs were taken using a HITACHI S800 FEG Microscope.



**Figure 3.** Ubx expression and function in the water-walking Microvelia americana. (A) During early embryogenesis, Ubx expression is absent from the mid-legs (L2) and appears first uniformly distributed in the rear-legs (L3). (B) During mid-embryogenesis, Ubx expression is now apparent in both mid- and rear-legs, with higher levels in the rear-legs concentrated in two main stripes (arrowheads). (C) During late embryogenesis, Ubx expression is now strong in three stripes in the rear-legs, and remains apparent in the mid-legs though still at much lower levels. (D) *Microvelia* fully developed embryo prior to hatching, showing the position of the tips of both mid- and rear-legs. (E) *Microvelia* fully developed embryo after *Ubx* RNAi treatment, showing that the tips of L2 and L3 in *Ubx* RNAi-treated embryo no longer reach their usual position shown in wild type due to shortening.

# Results

# UBX EXPRESSION AND FUNCTION IN REAR-LEGS IS CONSERVED BETWEEN TERRESTRIAL AND AQUATIC HETEROPTERANS

To test whether the role of Ubx in establishing the ancestral leg length plan (rear-legs longer than mid-legs) is conserved in a heteropteran species that adapted to life under water, we analyzed Ubx expression and function in the backswimmer Notonecta undulata. Notonecta, which uses its extremely elongated rear-legs as swimming oars under the water, is phylogenetically basal to both the terrestrial milkweed bug and the semi-aquatic bugs (Fig. 1) (Li et al. 2012). UbdA antibody (Kelsh et al. 1994) (see Methods) revealed that Ubx protein appears in early embryos, and persists throughout development in the rear-legs (Fig. 2A-B). We did not detect any expression in the mid-legs of Notonecta embryos. This pattern of Ubx expression that is restricted to the rear-legs of the T3 segment, is conserved relative to that of the terrestrial Oncopeltus (Mahfooz et al. 2007). However, unlike Oncopeltus where Ubx is expressed in the T3 tibia only, Ubx in Notonecta is expressed throughout all T3-leg segments, perhaps accounting for the extreme elongation of these legs. Parental RNAi knockdown in Notonecta resulted in the shortening of the rear-legs. This indicates that part of the role of Ubx in establishing the identity of T3 is to elongate the rear-legs (Fig. 2C-F). Therefore, in both aquatic and terrestrial Heteroptera, Ubx establishes the ancestral leg length plan, where the rear-legs are longer than the mid-legs, through its expression and elongating function in the rear-legs. Furthermore, the absence of Ubx expression and function in T2 is an ancestral feature common to aquatic and terrestrial Heteroptera, predating the evolution of the semi-aquatic insects.

## A NOVEL DOMAIN OF UBX EXPRESSION IN MID-LEGS EVOLVED IN THE COMMON ANCESTOR OF ALL SEMI-AQUATIC INSECTS

Over 200 million years ago, the common ancestor of the semiaquatic bugs transitioned from terrestrial life to life on the water surface (Andersen 1982; Li et al. 2012). This common ancestor had the ancestral leg length plan (mid-legs shorter than rear-legs) as inferred from the fossil record (Larsson 1978; Andersen 1979, 1982; Yao et al. 2011) and from basally branching families of extant Gerromorpha-Mesoveliidae, Hydrometridae, and Hebridae (Andersen 1982; Damgaard et al. 2005) (Fig. 1). A comparative analysis of leg length relative to body length revealed that species in the Geromorpha generally have longer mid- and rear-legs compared to their terrestrial and aquatic relatives (unpublished). We therefore investigated a possible role for Ubx in facilitating this morphological change, which is associated with their transition from land to water surface. We first examined Mesovelia mulsanti-a member of the basally branching Mesoveliidae (Fig. 1). Mesovelia are commonly found in transitional zones, where they walk on both ground and water surface (Andersen 1982). In early Mesovelia embryos, Ubx is expressed along the anterior-posterior axis of the rear-leg, and no staining could be observed in the mid-legs (Fig. 2G). Surprisingly, in later embryonic stages we detected Ubx expression also in the mid-legs (Fig. 2H). Ubx in late embryonic stages is now visible in both legs, although it remains stronger in the rear-legs



**Figure 4.** Ubx expression and function in the surface rowing veliid Rhagovelia and two gerrids Metrobates and Limnoporus. (A, G, and M) Ubd-A staining in early embryos showing that Ubx is expressed in both T2 and T3 in *Rhagovelia* (A) and *Metrobates* (G), but only in T2-legs in *Limnoporus* (M). (B, H, and N) expression in late embryos showing that Ubx is now expressed in both T2 and T3-legs in all three species. (C, I, and O) leg arrangement of late embryos of *Rhagovelia* (C), *Metrobates* (I), and *Limnoporus* (N). Open arrowheads indicate position of mid-leg tips and black arrowheads indicate rear-leg tips. (D, J, and P) effect of *Ubx* RNAi treatment on leg length in late embryos of *Rhagovelia* (D), *Metrobates* (I), and *Limnoporus* (P) as indicated by positions of the tips of mid- and rear-legs. (E, K, and Q) hatched nymphs of *Rhagovelia* (E) and *Metrobates* (K), and *Limnoporus* (Q) showing that relative leg length is established during embryogenesis. (F, L, and R) hatched nymphs showing the effect of *Ubx* RNAi on relative leg length in *Rhagovelia* (F) and *Metrobates* (L), and *Limnoporus* (R).

(Fig. 2H). Therefore, in *Mesovelia*, Ubx appears first in the rear-legs and later in the mid-legs during embryogenesis. These differences in the timing of Ubx expression between the midand rear-legs are consistent with the difference in length between these legs. This finding is surprising because, like the terrestrial relative *Oncopeltus* and the aquatic relative *Notonecta*, *Mesovelia* rear-legs are longer than the mid-legs. Therefore, although Ubx gained a new domain of expression, it was not associated with a reversal in relative mid- to rear-leg length.

Parental RNAi against *Ubx* in *Mesovelia* revealed that both mid- and rear-legs of treated embryos are shorter than control embryos (Figs. 2I–L, 5), demonstrating a role of Ubx in lengthening both mid- and rear-legs. However, we also found that the effect of *Ubx* RNAi is three times greater in rear- than in midlegs (Fig. 5). This result suggests that Ubx lengthens the rear-legs more than it lengthens the mid-legs in this species, also consistent with the earlier appearance of Ubx in T3-legs compared to T2-legs during embryogenesis. This novel deployment of Ubx in T2 segment with its ancestral role in increasing leg length is absent in aquatic and terrestrial Heteroptera and coincides with the invasion of the water surface by the ancestors of the semi-aquatic bugs.

# CHANGES IN UBX EXPRESSION AND FUNCTION IN MID- AND REAR-LEGS UNDERLIE THE EVOLUTION OF THE DERIVED LEG PLAN AND WATER SURFACE ROWING

Subsequent to the invasion of the water surface, semi-aquatic insects evolved surface rowing as a specialized mode of locomotion



**Figure 5.** Effect of Ubx RNAi on individual leg segments during embryogenesis across aquatic and semi-aquatic bugs. Light gray bars indicate wild type and lack of significance in *Ubx* RNAi on segment length. Green indicates Ubx's role in increasing and red in decreasing the length of individual leg segments. Asterisks indicate statistical significance (P < 0.05). In *Mesovelia*, the reduction in L2 tarsus length in *Ubx* RNAi was 6% (light green, plus sign), but only approached significance (P = 0.078). Despite the fact that we could not detect Ubx in any segments of *Notonecta* L2, we saw a small but significant effect (P = 0.037) of *Ubx* RNAi on L2 tarsus length (Light green, plus sign). We suspect that this is the result of allocation adjustments in the *ubx* RNAi phenotypes. Number of embryos included in leg length measurements is N = 10 in all species except *Mesovelia* where N = 7. Standard error bars are small because embryos included in this analysis mostly show strong unambiguous *Ubx* phenotype.

on the open water in lakes, streams, and oceans. Surface rowing is thought to have evolved independently in two families; multiple times in the paraphyletic Veliidae and once in the common ancestor of the Gerridae (Andersen 1982; Damgaard et al. 2005; Damgaard 2008b). In the surface rowing Gerrid, *Gerris buenoi*, Ubx establishes the derived leg length plan (mid-legs longer than rear-legs) during embryo development by lengthening the midlegs and shortening the rear-legs (Khila et al. 2009). All surface rowers share this derived leg plan, yet there is a great diversity in leg lengths and niche specialization among them (Fig. 1).

To investigate the role of the *Ubx* gene in generating this diversity, we examined two members of the Veliidae and two additional members of the Gerridae (Fig. 1). The derived veliid *Mi*-

*crovelia* is a surface walker that shares a similar niche (land–water boundary) and ancestral leg length plan (mid-legs shorter than rear-legs) with the basally branching *Mesovelia*. Similar to our results in *Mesovelia*, Ubx is expressed in and is required to lengthen both mid- and rear-legs in *Microvelia* embryos (Fig. 3). Therefore, despite the phylogenetic distance between *Mesovelia* and *Microvelia*, the conserved ecological niche occupied by these two species may have favored the retention of Ubx's role in elongating both legs without reversing their relative length. We then examined the veliid *Rhagovelia* and the two gerrids *Limnoporus* and *Metrobates*, all of which share surface rowing as a novel mode of locomotion, and a further elongation of the mid-legs, which function as oars (Andersen 1982; Damgaard et al. 2005; Damgaard

2008b). We detected Ubx expression in both mid- and rear-legs at early and late developmental stages in the veliid Rhagovelia (Fig. 4A-B) and the gerrid Metrobates (Fig. 4G-H). This early timing of Ubx expression is consistent with the great length of some leg segments in these two species, especially rear-leg tibia in Rhagovelia (Fig. 4E) and rear-leg femur in Metrobates (Fig. 4K). In the other gerrid Limnoporus however, we found that Ubx appears first in the mid-legs, and then later during embryogenesis in rear-legs (Fig. 4M-N). These changes in the timing of Ubx expression in the mid- and rear-legs distinguish the surface-rowing species Rhagovelia, Metrobates, and Limnoporus from the water walking Mesovelia and Microvelia, and are consistent with the evolution of the derived leg length plan. Ubx RNAi in the three species resulted in shorter mid-legs, but longer rear-legs compared to control embryos (Fig. 4C-F, I-L, O-R). Together, these results suggest that the derived leg length plan of surface-rowing species is established through an advancement of the timing of Ubx expression in the mid-legs to elongate them, and reversal of its function in the rear-legs to shorten them. These data suggest that the transition from walking in water-ground boundary niche to exclusive water surface rowing may have selected for changes in Ubx expression and function that establish the derived leg length plan of surface rowers.

# UBX EXPRESSION AND FUNCTION IS ASSOCIATED WITH FINE SCALE INTER-SPECIFIC VARIATION IN RELATIVE SEGMENT LENGTH OF THE REAR-LEGS

Although mid-legs are generally longer than rear-legs in surface rowing semi-aquatic bugs, within this derived leg plan there is substantive variation in the lengths of each leg segment along the proximal-distal axis (Figs. 1, 4, 5) (Matsuda 1960; Andersen 1982). This diversity in the relative length of leg segments is thought to reflect the adaptation to the distinct water surface niches occupied as the semi-aquatic bugs radiated (Andersen 1976, 1979, 1982). Surprisingly, we discovered that Ubx expression and function is also associated with these finer scale inter-specific differences in the rear-legs. In the veliid Rhagovelia, Ubx RNAi elongates the tarsus, shortens the tibia, and has no significant effect on the length of the femur in the rear-legs (Fig. 5). Therefore in this species, Ubx shortens the rear-legs primarily by shortening the tarsus (Fig. 5); Ubx retains its ancestral elongating function in the tibia and has no significant effect on the femur (Fig. 5). In contrast in the gerrid Metrobates, Ubx shortens the rear-legs by dramatically shortening the tarsus and the tibia, while retaining its ancestral elongating function in the femur. In the two Gerrids, Gerris (Khila et al. 2009) and Limnoporus, Ubx shortens the rear-legs by shortening all three segments (Fig. 5). These opposing functions of Ubx in regulating the length of individual segments in the same leg within and between species may have been key to generating finer scale adaptations to the variety of finer scale ecological niches occupied by each of these species. These results also support the idea that *Ubx* regulates differences in morphology by modifying each specific segment within the third leg independently (Akam 1998a; Stern 1998, 2003). It is this independent and specific nature of *Ubx* regulation that has repeatedly enabled the semi-aquatic insects to exploit a wide variety of water surface niches.

# UBX CONTROLS DIFFERENCES IN OTHER ADAPTIVE MORPHOLOGICAL TRAITS BETWEEN T2 AND T3 SEGMENTS

Ubx in insects is thought to independently control specific morphological characters within a segment, such that the collective sum of these characteristics imparts a given segment identity (Castelli-Gair and Akam 1995; Akam 1998a,b; Castelli-Gair 1998; Stern 1998; Roch and Akam 2000; Rozowski and Akam 2002; Mahfooz et al. 2007). To determine how Ubx may have additionally contributed to the diversification of the group upon adaptation to water surface life, we examined other fine-scale morphological characteristics that are affected by Ubx RNAi. In all Gerromorpha, the legs are equipped with combs that are used to groom the hydrophobic hair layers that form the contact surface between legs and water (Andersen 1982). The role of these grooming combs is critical as they keep the hydrophobic hairs clean, therefore preventing the insects from wetting and sinking (Andersen 1976, 1982). There is a great deal of variation, however, in terms of number and location of these combs within segments among species. In the basally branching Mesovelia, the forelegs bear two combs at the base of the tibia, the mid-legs one comb, while there is no comb in the rear-legs (Fig. 6A-C). In Ubx RNAi Mesovelia embryos, the combs on the fore- and mid-legs remain unaffected in number and morphology (Fig. 6E-F). However, the rear-legs now develop one comb on the base of the tibia, reminiscent to that found in the mid-legs (Fig. 6G). An ectopic leg, characteristic of Ubx RNAi (Angelini et al. 2005), develops on the first abdominal segment (compare Fig. 4D and H). The appearance of the comb suggests that Ubx parental RNAi caused at least a partial homeotic transformation of T3 to T2. These results have multiple implications for the interpretation of Ubx's role in imparting segment identity. First, Ubx RNAi is specific and does not affect the first legs consistent with the absence of Ubx expression in T1 segment. Second, in Ubx knockdown, T2 does not assume T1 identity as indicated by the difference in the number of combs. Finally in Mesovelia, Ubx represses comb formation in the rearlegs but not in the mid-legs despite Ubx expression in both legs. In the derived surface rower Limnoporus, unlike the basally branching walker Mesovelia, both mid-legs and rear-legs have one comb (Fig. 7A-B). Interestingly, Ubx RNAi in Limnoporus does not affect comb development in any of the legs (Fig. 7C–D), suggesting that in derived rowing species where Ubx function in T3 has been



**Figure 6.** Grooming combs in control and Ubx RNAi late embryos of *Mesovelia mulsanti*. *Mesovelia* forelegs bear two combs (arrows in A), the mid-legs bear a single comb (arrow in B), and the rear-legs do not have any combs (C). Ubx RNAi does not affect the combs either in the forelegs (E) or in the mid-legs (F). However, an ectopic comb develops on the rear-legs following Ubx RNAi (G). Note that the first thoracic segment now develops an ectopic limb (thick arrows in G), characteristic of Ubx RNAi in insects (compare D and H).



**Figure 7.** Grooming combs in control and *Ubx* RNAi late embryos of *Limnoporus dissortis*. (A) and (B) both mid-legs and rear-legs bear one comb a the base of the tibia (arrows). (C) and (D) *Ubx* RNAi does not affect any of these combs (arrow).

reversed to shorten the length of the rear-legs, Ubx has lost its repressive role on comb development. Therefore, the differential development of the combs between T2 and T3 is a characteristic that is no longer under Ubx control in derived Gerromorpha, and is now shared by T2 and T3 segments. Collectively, our results suggest that the evolution of these fine-scale morphological differences between different segments and species of semi-aquatic insects were facilitated by Ubx action that may independently control these multiple and small-scale differences between segments during development and evolution of the semi-aquatic insects.

# Discussion

The invasion of the water surface and subsequent radiation of semi-aquatic insects required the evolution of new means of locomotion, which in turn required both large and small evolutionary change in the locomotory appendages (Andersen 1976). Collectively, our results suggest that evolutionary change in large-scale domains of Ubx expression, reversals in its function, and smaller scale spatial-temporal variation in expression are associated with major changes in appendage plans, and more minor changes in segment lengths and other adaptive structures. Our ability to perform functional analyses in a comparative context, and on a broad scale, was necessary for reconstructing multiple unexpected evolutionary steps and paths leading to diversification in this group.

The extent to which these stepwise changes represent divergent, parallel, or convergent evolution depends upon the level at which one asks the question (Abouheif 1997; Abouheif et al. 1997; Wray and Abouheif 1998). For example, functionally, the veliids and gerrids have convergently evolved the elongated midlegs used to row on the water surface (Andersen 1982; Damgaard et al. 2005; Damgaard 2008b). Moreover, the evolution of Ubx expression and function are involved in broadly similar ways in



**Figure 8.** Evolutionary transitions in Ubx expression and function and its role in leg diversification and habitat specialization of different Gerromorphan species. Diagrams represent species examined in this study, except *Oncopeltus* (Mahfooz et al. 2007) and *Gerris* (Khila et al. 2009). Mode of locomotion, niche occupation, and the role of Ubx in appendage diversification are indicated.

this elongation, suggesting parallel evolution. Yet, variation in which segments of the rear-legs are elongated or shortened, suggests divergent paths to this state. Thus the *Ubx* gene was involved in both convergent and divergent evolution of relative leg length, through similar developmental mechanisms, depending only upon at which level one looks.

The molecular genetic mechanisms underlying these changes in Ubx expression and function observed here are under investigation. In the fly Drosophila, Ubx expression domain is restricted to the posterior thoracic segments through the action of a variety of gap and segmentation genes (Qian et al. 1993; Wu et al. 2001; Marques-Souza et al. 2008). Therefore, the expansion of Ubx expression to the second pair of embryonic legs in the common ancestor of the Gerromorpha, and the subsequent change in its timing of expression in some Veliidae and Gerridae, may be due to changes in its interactions with these upstream regulators. Furthermore, Ubx protein is known for its capacity to control and interact with a variety of target genes and cofactors in different tissues at different stages of development (Merabet et al. 2007; Pavlopoulos and Akam 2011; Saadaoui et al. 2011). Therefore, the interaction between Ubx and distinct cofactors and target genes along the proximal-distal leg axis may be driving the rapidly changing role of Ubx in controlling leg length between species. The substantive changes in the response of the legs and leg segments across the semi-aquatic bugs also suggest changes in the landscape of Ubx downstream target genes in these lineages.

This work represents an example where an upstream regulator, combined with the evolutionary diversification of its target gene networks, can profoundly shape adaptive morphological traits associated with species radiation (Stern and Orgogozo 2008).

Our results link changes in Ubx expression and function with the transitions of the semi-aquatic insects into a novel ecological habitat, and the adaptive radiation that followed. These findings map the genetic basis of appendage diversification within an ecological model proposed by Andersen (Andersen 1982). In this model (Fig. 8), Andersen describes a series of transitions between three distinct adaptive zones, defined on the basis of the substrate upon which Geromorphans lived (Fig. 8). The first ancestors occupied solid substrates covered by a thin film of water, followed by invasion of the intersection zone by species such as Mesovelia and Microvelia, primarily adapted to life in this transitional zone between terrestrial and water surfaces. Finally, there was a radiation into the variety of open water niches by species specialized in rowing, such as Gerris, Limnoporus, Rhagovelia, and Metrobates (Andersen 1982). We have identified changes in the role and regulation of a key developmental gene that are associated with these transitions, and mapped these changes along the various paths followed during the radiation of the group (Fig. 8).

Among the multiple adaptations accompanying the transition from ground to water surface (Figs. 1 and 8) was a notable ability to circumvent the constraints imposed by the hydrodynamics of fluid propulsion (Andersen 1976; Hu et al. 2003; Hu and Bush 2010). The novel deployment of Ubx in T2-legs increased their length relative to body length in the ancestors of the Gerromorpha. Increased leg length relative to body length is expected to increase the amplitude of the stroke of the propelling legs, thereby allowing more efficient propulsion on the water surface (Andersen 1976, 1982; Hu et al. 2003; Hu and Bush 2010). In support of this hypothesis, high-speed video imaging of Mesovelia locomotion demonstrated that the stroke of Mesovelia mid-legs and rear-legs cover greater amplitude during water walking compared to ground walking (not shown). Furthermore, during water walking, the amplitude of mid-leg movements (the pair of legs whose length was increased through the novel deployment of Ubx) is greater than that of the fore- and the rear-legs. This suggests that a substantial part of the propulsion during water walking is generated by the mid-legs. We also observed similar biomechanics in the veliid Microvelia, which occupies a similar niche as Mesovelia. In the light of these observations, it is possible that elongated mid-legs, through the novel deployment of Ubx in T2, may have increased locomotion efficiency on water surface. We therefore propose that the gain of Ubx expression and function in the mid-legs may have been a key change at the base of the Gerromorpha that enabled the invasion of water surface by the semi-aquatic insects.

Finally, the novel deployment and function of Ubx in the mid-legs early in the evolution of the Gerromorpha may have provided the genetic potential for further changes in Ubx regulation, including changes in the timing of expression in the mid-legs and reversals in function in the rear-legs. This, together with the role of Ubx in generating fine-scale adaptive structures such as the grooming combs, may have facilitated the specialization and diversification of the group within the vast array of open water surfaces (Andersen 1982). Therefore, key developmental genes can both accompany invasion of new ecological habitats and fuel adaptive radiation.

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# Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Figure S1. Sequence of *Ubx* across species used in this study, and that of Drosophila for comparison.

Table S1. Primers used to clone Ubx across species.

 Table S2. T7 primers used to synthesize Ubx double stranded RNA template.

Table S3. Ubx RNAi experiment across species.