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The Crustacean *Parhyale hawaiensis*: A New Model for Arthropod Development

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INTRODUCTION

The great diversity of arthropod body plans, together with our detailed understanding of fruit fly development, makes arthropods a premier taxon for examining the evolutionary diversification of developmental patterns and hence the diversity of extant life. Crustaceans, in particular, show a remarkable range of morphologies and provide a useful outgroup to the insects. The amphipod crustacean *Parhyale hawaiensis* is becoming established as a model organism for developmental studies within the arthropods. In addition to its phylogenetically strategic position, *P. hawaiensis* has proven to be highly amenable to experimental manipulation, is straightforward to rear in the laboratory, and has large numbers of embryos that are available year-round. A detailed staging system has been developed to characterize *P. hawaiensis* embryogenesis. Robust protocols exist for the collection and fixation of all embryonic stages, in situ hybridization to study mRNA localization, and immunohistochemistry to study protein localization. Microinjection of blastomeres enables detailed cell-lineage analyses, transient and transgenic introduction of recombinant genetic material, and targeted knockdowns of gene function using either RNA interference (RNAi) or morpholino methods. Directed genome sequencing will generate important data for comparative studies aimed at understanding *cis*-regulatory evolution. Bacterial artificial chromosome (BAC) clones containing genes of interest to the developmental and evolutionary biology communities are being targeted for sequencing. An expressed sequence tag (EST) database will facilitate discovery of additional developmental genes and should broaden our understanding of the genetic controls of body patterning. A reference genome from the related amphipod crustacean *Jassa slatteryi* will shortly be available.

RELATED INFORMATION

The National Human Genome Research Institute (NHGRI) maintains a list of approved sequencing targets (http://www.genome.gov/10002154). *J. slatteryi* is an approved sequencing target of the NHGRI's Large-Scale Genome Sequencing Program. The proposal (which was accepted) to sequence five species within the Ecdysozoa, including *J. slatteryi*, can be found at http://www.genome.gov/Pages/Research/Sequencing/SeqProposals/EcdysozoaProposalFinalPDF.pdf.

BACKGROUND INFORMATION

The amphipod *P. hawaiensis* (Dana 1853) is a crustacean species that is particularly well suited for developmental, genetic, and evolutionary analyses, and it has the potential to fill an important taxonomic gap in current comparative studies. Commonly referred to as "beachhoppers" or "scuds," amphipods are malacostracan crustaceans and are therefore closely affiliated with more familiar

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crustacea such as krill, lobsters, and crabs. Within the taxonomic group Crustacea, amphipods rank as one of the most ecologically successful and speciose extant orders, and they occur in nearly all known marine, fresh, and brackish water environments as well as in high-humidity terrestrial ecosystems such as tidal zones, coastal flood plains, and forest leaf litter (Barnard and Karaman 1991; Lindeman 1991; Vinogradov et al. 1996; Kamaltynov 1999; Sherbakov et al. 1999; Vainola and Kamaltynov 1999; Poltermann et al. 2000; Sheader et al. 2000; Gasca and Haddock 2004). This ecological diversity is matched by a high level of morphological diversity. Several thousand amphipod species have been described, with several new species descriptions occurring every year.

Several aspects of the life history of *P. hawaiensis* make this particular species amenable to many types of classical and modern laboratory analyses and techniques. *P. hawaiensis* is a detritovore that has a circumtropical, worldwide, intertidal, and shallow-water marine distribution (Shoemaker 1956; Barnard 1965), and it may occur as a species complex (Myers 1985). It has been reported to aggregate in large populations (>3000/m^2) on decaying mangrove leaf material in environments subjected to rapid changes in salinity (Poovachiranon et al. 1986). The ability to tolerate rapid temperature and osmotic changes characteristic of its preferred shallow-water habitat allows this species to thrive under typical laboratory conditions.

Several characteristics of *P. hawaiensis* have made it particularly amenable to embryological and molecular genetic manipulation. Females produce embryos every 2 wk once they reach sexual maturity. Embryogenesis is relatively short, lasting ~10 d at 26°C. Close examination of the embryonic development of *P. hawaiensis* has produced the most detailed staging system for any crustacean (Browne et al. 2005). Complete embryogenesis has been divided into 30 discrete stages, which are readily identifiable in living animals or by means of common molecular markers in fixed specimens. As direct developers, hatchlings possess a complete complement of segments and appendages morphologically similar to those of adult animals. Females normally brood the embryos in a ventral brood pouch. Embryos can be rapidly and easily removed from the brood pouch and maintained in seawater. Eggs can be collected and hatched individually, and the mature animals can subsequently be used in pairwise sister-brother or mother-son matings to generate inbred lines. Fertilized eggs can be removed from females before their first cleavage and are sufficiently large to perform microinjections (Gerberding et al. 2002) and blastomere isolations (Extavour 2005) with relative ease. Developing *P. hawaiensis* embryos are optically clear, allowing for both detailed microscopic analyses in situ and the use of fluorescently tagged tracer molecules in live embryos. The yolk, although opaque, is sequestered early in development at the center of the developing egg and then later to the developing midgut of the embryo. In addition, early cleavage is holoblastic (total), allowing the fates of individual early cells to be explored through experimental manipulation (Gerberding et al. 2002; Extavour 2005).

In addition to being a robust laboratory performer amenable to a wide variety of experimental manipulations, *P. hawaiensis* occupies a phylogenetic position that has important consequences for our understanding of the evolutionary processes responsible for the remarkable radiation of insects and crustaceans. Several recent studies have reexamined evolutionary relationships among the major groups of arthropods. The data suggest two possible relationships between insects and crustaceans. One possibility is that the two groups are sister taxa (Boore et al. 1995, 1998; Friedrich and Tautz 1995; Eernisse 1997; Giribet et al. 2001). The other possibility is a "Pancrustacea" clade, in which the insects branch from within branchiopod crustacea (Regier and Shultz 1997; Hwang et al. 2001). In this scenario, insects represent a terrestrialized branch of crustaceans, and branchiopod crustaceans, which include *Artemia* and *Daphnia*, are more closely related to insects than are malacostracan crustaceans such as crabs, isopods, and amphipods. In either case, malacostracan crustaceans such as amphipods provide a key group for understanding arthropod evolution.

Many of the preceding general considerations of *P. hawaiensis* as well as the following sketches of morphology and embryonic development were extracted and condensed from a more extensive staging paper, which can be consulted for further details (Browne et al. 2005).

SOURCES AND HUSBANDRY

A population of *P. hawaiensis* was originally obtained in 1997 from the marine filtration system of the John G. Shedd Aquarium in Chicago. From this founding population, a laboratory culture of *P. hawaiensis* was successfully established and has subsequently been dispersed to other laboratories in the United States and Europe. Laboratory breeding colonies are maintained in shallow plastic trays

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(47 × 33 × 12 cm) at 26°C. The containers should remain covered, and the water should be at least 4 in. deep to prevent rapid changes in salinity due to evaporation. A thin (1/4-in.) layer of calcium carbonate gravel spread over the bottoms of tanks serves as a pH buffer as well as a substrate for the animals. Adequate circulation is essential to allow proper biological filtration and gas exchange. This is best achieved by using an airstone and/or a submersible water pump. Illumination provided by fluorescent strip lighting is placed on a day/night cycle of 14/10 h. Artificial saltwater (Tropic Marin) is prepared to a specific gravity of 1.018-1.022, and after the salt has fully dissolved, it is aerated overnight with an airstone before use. Filtered natural seawater can also be used. Trays should receive a 50% water change each week. Optimal growth is obtained by heating the aquarium room to 26°C, although animals can be grown at 20°C-26°C. *P. hawaiensis* is maintained on a diet of *Ascophyllum nodosum* kelp granules (Starwest Botanicals) soaked in seawater and a 1:1:1 liquid mixture of fatty acids (Selcon), plankton (MarineSnow), and vitamins (MultiVit, Hawaiian Marine).

Once a tank is established, it is typically only necessary to monitor salinity and temperature. When a tank is first set up, however, it is important to check several other parameters. To maintain an ideal pH range of 8.0-8.4, artificial seawater should be made from deionized water and changed weekly, and a calcium carbonate substrate should be used. Failure to clean the tank sufficiently and/or poor gas exchange will lower the pH. Ammonia and nitrites are the natural products of waste protein decomposition, and even small amounts are toxic to crustaceans. Test kits are available from aquarium supply stores, and detectable levels of either substance indicate that the biological load of the tank exceeds its capacity to process waste. Decreasing the population density and/or changing the seawater should solve the problem. Although nitrates are far less toxic than other nitrogenous compounds, they fuel algae growth, which can be a nuisance. In a well-maintained tank, nitrates should be close to zero and always maintained at <10 ppm.

Smaller tanks can be maintained on individual laboratory benches. Lidded plastic food storage containers aerated with small airstones and external aquarium pumps are adequate for this task. Typically, bench-top working populations of *P. hawaiensis* are sustained solely on a diet of baby carrots. The lower population densities in these bench-top colonies together with carrots as a food source results in very large animals. Sexually mature pairs collected from these bench-top tanks routinely result in gravid females with broods of 20-30 embryos. Large broods facilitate the collection of synchronous populations of embryos. For experimental manipulation of embryos, sexually mature pairs in amplexus are isolated in small plastic food storage containers with seawater.

Within 12-24 h, most of the animals will have separated. The rate of separation may be decreased by keeping pairs at 18°C. Separation occurs as females shed their old cuticle. Once the molt is complete, mature oocytes are released through the oviducts, fertilized, and deposited in the female's ventral brood pouch. Gravid females are anesthetized with clove oil, and the embryos are removed from the brood pouch (see **Fixation and Dissection of *Parhyale hawaiensis* Embryos** [Rehm et al. 2009a]). Following embryo collection, females are allowed to awaken and are then returned to a bench-top tank. Low-density working populations can be supplemented, as needed, from the larger tanks.

Embryos should be serially rinsed in three to four washes of seawater in medicine cups, after which they may be placed in filter-sterilized seawater (22-µm Millipore filter) in 35-mm tissue culture dishes (Falcon) and maintained in a high-humidity chamber at 26°C until they reach specific time points for manipulation, dissection, and/or fixation. Under these conditions, embryonic development will proceed as described by Browne et al. (2005). Adult females should be allowed to awaken for at least 30 min in 500 mL of seawater before being returned to an aerated bench-top tank. Amphipods and their embryos must not be left too long in clove oil.

RELATED SPECIES

J. slatteryi and *P. hawaiensis* are relatively closely related amphipods. From cDNA sequence comparisons, we estimate that about the same molecular distance separates these two amphipods as separates *Drosophila melanogaster* and *Drosophila virilis*. The embryonic development patterns of *J. slatteryi* and *P. hawaiensis* appear to be largely identical; if not for the smaller size of early *J. slatteryi* embryos, they would be difficult to distinguish morphologically from those of *P. hawaiensis*. What does distinguish *J. slatteryi* is the relatively small size of its genome. At 690 Mb, the *J. slatteryi* genome is only 10 Mb larger than the smallest recorded genome within the malacostracan crustaceans. Unlike *P. hawaiensis*, however, *J. slatteryi* has proved to be difficult to culture in the laboratory. Obtaining wild-caught *J. slatteryi* adults is reasonably straightforward, because they live along the

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Northern California coastline and in the San Francisco Bay. Efforts are under way to develop an inbred laboratory population of *J. slatteryi*.

J. slatteryi is an approved sequencing target of the National Human Genome Research Institute's (NHGRI) Large-Scale Genome Sequencing Program (http://www.genome.gov/10002154 and http://www.genome.gov/Pages/Research/Sequencing/SeqProposals/EcdysozoaProposalFinalPDF.pdf. The *J. slatteryi* genome will be sequenced with fivefold coverage by the Broad Institute at the Massachusetts Institute of Technology. In addition, *J. slatteryi* BAC and cDNA libraries are being constructed. The Broad Institute will generate paired-end reads from 100,000 embryonic cDNAs and sixfold BAC end sequencing to complement the *J. slatteryi* genome sequence.

USES OF THE *P. HAWAIENSIS* MODEL SYSTEM

Although *P. hawaiensis* has been used sporadically for toxicological and ecological studies throughout the years, a concerted effort has recently been made to establish the amphipod *P. hawaiensis* as a new crustacean model organism for studying the relationship between development and evolution (Browne et al. 2005).

Body Plan of *P. hawaiensis*

The body plan of *P. hawaiensis* (Fig. 1) is organized around a series of repeating segmental units along the anterior-posterior (AP) axis. Several synapomorphic characteristics clearly unite the Amphipoda

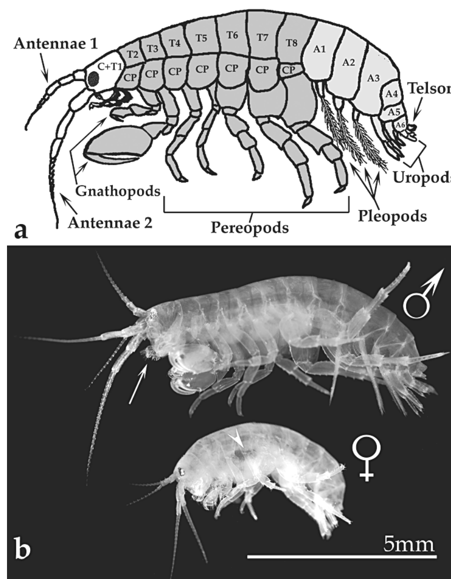

FIGURE 1. The *P. hawaiensis* body plan. (*a*) Schematic diagram of the adult body plan. By convention, the (C) head (white) consists of the anteriormost six segments (termed the "cephalon") plus the first segment of the thorax (T1). All head segments posterior to the second cephalic segment bear a pair of appendages. From anterior to posterior, these appendages are as follows: antennae 1, antennae 2, mandibles, first maxillae, second maxillae, and the maxillipeds of the first thoracic segment (T1). (Dark gray) The pereon, composed of T2-T8. Each thoracic segment of the pereon possesses a pair of appendages, and the most proximal element of each appendage, the coxa, has a dorsal branch that is compressed and expanded into a structure called the coxal plate (CP). Appendages T2 and T3 are termed "gnathopods" and are distinctly subchelate (clawed). The T3 gnathopod is sexually dimorphic: In mature males, it is greatly enlarged (compare male to female in *b*). Segments T4-T8 possess appendage pairs termed "pereopods" that function primarily for locomotion. (Light gray) Abdominal segments A1-A6. A1-A3 form the pleon, and each bears a pair of appendages termed "pleopods." The final three segments of the abdomen (A4-A6) form the urosome, and each of these segments bears a pair of appendages termed "uropods." At the very posterior is the telson, which is a cleft flap of cuticle just posterior and dorsal to the anus. (*b*) Sexually mature animals possess several dimorphic characters. Males are larger than females, and T3 appendages are greatly enlarged in males. (Arrowhead) Females possess a ventral brood pouch in which they incubate their eggs until hatching. (*Top*, arrow) All amphipods retain a highly compact arrangement of mouthparts termed the "buccal mass."

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(Schram 1986; Schmitz 1992). The orientation (pointed anterior) of T4 and T5 walking appendages relative to those of T6-T8 (pointed posterior) is responsible for the name of the group: "amphipod." Other recognizable characteristics are lateral compression of the body, sessile compound eyes, and large coxal plates attached dorsally to the base of thoracic appendages. As is the case in most amphipods, the *P. hawaiensis* cephalon (head) is composed of the six anteriormost segments. The anteriormost preantennal segment bears no paired appendage. The remaining five segments do possess paired appendages. From anterior to posterior, the paired appendages of the head are the uniramous first antenna (An1), uniramous second antenna (An2), gnathobasic mandibles (Mn), biramous first maxillae (Mx1), and the biramous second maxillae (Mx2). In addition, the first thoracic segment (T1) is fused to the cephalon. The T1 appendage pair, the maxillipeds, are triramous, fused at their base and extensively modified to assist in feeding. There is a close arrangement of the gnathal appendages, including the maxillipeds, in a basket shape around the mouth to form a highly compact buccal mass. The next seven segments of the thoracic region, T2-T8, articulate independently and bear a pair of appendages. Appendages T2-T5 of sexually mature females possess an additional ventral appendage branch, termed an "oosteogite," which interlocks to form the protected ventral brood pouch into which fertilized eggs are shed and incubated until hatching. The T2 and T3 appendages, termed "gnathopods," are subchelate and function in grasping and mating. The T3 appendage is sexually dimorphic (larger on mature males). The T4-T8 appendages, functioning in locomotion, are termed "pereopods."

The abdominal region is composed of the next six segments and is divided into two regions: segments A1-A3 constitute the pleon and A4-A6 the urosome. Each abdominal segment bears a pair of biramous appendages. The A1-A3 appendages are termed "pleopods" and are used both for swimming and for drawing water across the ventrally located thoracic gills. Each pair of pleopods is fused at its base along the ventral midline of the animal and is highly setose. The appendages of A4-A6 are termed "uropods." These appendages are reduced and thickened and bear several stout spikes along their proximodistal axes. The terminal structure, the telson, is cleft and reduced in size relative to comparable structures of other types of crustaceans.

Developmental Staging of *P. hawaiensis*

Parhyale embryogenesis is briefly described here with particular attention to morphological signposts that are helpful for staging embryos. Key time points of *P. hawaiensis* development are further illustrated by means of live bright-field and matching post-fixation DAPI (4′-6-diamidine-2′-phenylindole, a fluorescent stain for nuclei) images, as well as bright-field anti-Engrailed stains. For more detailed descriptions and additional imagery, consult Browne et al. (2005). All developmental times refer to embryos maintained at 26°C.

Mating and Fertilization

Sexually mature male and female *P. hawaiensis* form mating pairs in which males grasp and hold smaller females with their second thoracic appendages (T2, gnathopods) until mating occurs. The pairs remain in pre-mating amplexus until the female molts, at which point the male deposits sperm into the female's paired oviducts and releases her. Before the female's new cuticle hardens, she sheds her eggs into a ventral brood pouch through two bilaterally symmetric oviducts, fertilizing them in the process.

Stages 1-4: Oocyte to Eight-Cell Embryo

Stages 1-4 are illustrated in Figure 2. Within 1 h of being deposited into the brood pouch, eggs assume an elliptical shape as their outer chorion shells harden. The oocyte/yolk mass usually appears purplish and is consistent for all embryos within a given brood. After 4 h (26°C), a total first cleavage occurs that is meridional and perpendicular to the egg's long axis, resulting in a two-cell embryo. A second total cleavage that is meridional and parallel to the long axis of the egg results in a four-cell embryo. At 8 h, a highly unequal and equatorial third cleavage occurs, perpendicular to the first two planes of cleavage, resulting in an eight-cell embryo with four distinct macromeres (Mav, Er, El, and Ep) and four micromeres (g, mr, ml, and en). An interesting and unusual feature of *P. hawaiensis* embryogenesis is that each cell of an eight-cell embryo already bears a distinct fate (Gerberding et al. 2002). At the eight-cell stage, individual identities of each macromere and micromere can be determined by size, shape, refringency, and relative position of the blastomeres.

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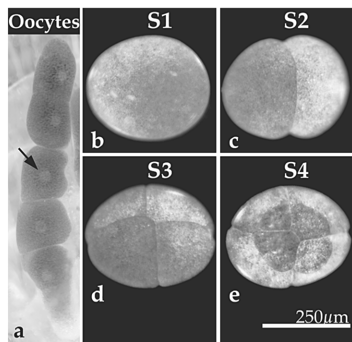

FIGURE 2. *P. hawaiensis* oocytes and stages 1-4 (S1-S4). (*a*) Right ovary with six mature oocytes (anterior *up*, posterior *down*). (Arrow) The oocyte nucleus is visible as a white oval in the yolk-laden oocyte. (*b*) Stage 1 (S1); single uncleaved cell. (*c*) Stage 2 (S2); the first cleavage plane is meridional and slightly asymmetric. (*d*) Stage 3 (S3); the second cleavage plane is meridional and also slightly asymmetric. (*e*) Stage 4 (S4); vegetal view, the third cleavage plane is equatorial and highly asymmetric, generating an eight-cell embryo composed of four macromeres and four micromeres.

Stages 6-8: Blastoderm, Gastrulation, and Germ-Disc Condensation

Stages 6-8 are illustrated in Figure 3. By 12 h of development, embryos reach the "soccer ball" stage (stage 6). The yolk has been displaced toward the center of the egg, which is largely devoid of nuclei. Similarly sized cells with distinctive whitish cytoplasm are distributed evenly along the egg periphery. The first significant cell migrations are associated with early gastrulation, and they occur between 12 and 18 h of development. By stage 7 (18 h), a majority of the cells have aggregated in the presumptive ventral end of the embryo, where they assume a hexagonal shape and begin forming a tight sheet. A second distinct cluster of cells, the "rosette," derives from Mav and g descendants. The rosette marks the position of the first group of cells that will move beneath the peripheral surface of the embryo. The rosette further indicates the future anterior end of the embryonic AP axis and, once it has migrated under the ectoderm, provides the first evidence of multiple germ layers. A third group of cells remains relatively stationary scattered across the remaining embryo. As these cells continue to proliferate, their descendants either migrate into the condensing germ disc or remain stationary outside of the embryo proper. At stage 8 (25 h), embryos are defined by germ-disc condensation, the gradual aggregation of cells along the anterior ventral region of the egg. Rosette cells are now positioned under the ectoderm anlagen and begin to move to their final positions. A depression in the germ disc becomes visible, directly above the rosette cells. This is shortly followed by the migration of ml and mr micromere descendants, under the ectoderm anlagen regions, that flank the rosette. The further development of the germ disc is characterized by continued cell proliferation and a general reduction in cell size.

Stages 11-18: Germ-Band Formation and Elongation

Some of stages 11-18 are shown in Figure 4. The germ disc continues to grow and organize via proliferation as well as the recruitment of cells from both posterior and lateral positions. By stage 11 (60 h), cells within the germ disc begin organizing into head lobes and germ band, fixing the relative

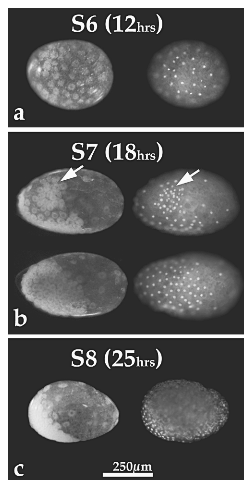

FIGURE 3. *P. hawaiensis* stages 6-8. Incident light bright-field photos of (*left*) living embryo and (*right*) matching DAPI image after fixation. (*a*) Stage 6 (S6), "soccerball" stage; most cells are approximately the same size and evenly distributed around the egg periphery. (*b*) Stage 7 (S7), "rosette" stage; same embryo shown in two orientations. (*Top* image pair, lateral view) Position and distribution of cells and their nuclei in the rosette. (White arrows) Indicate the position of rosette cells and nuclei. (*Lower* image pair, animal view) The large cluster of cells and nuclei migrating into the position of the future germ disc. (There is a slight rotation between bright-field and DAPI images.) (*c*) Stage 8 (S8); formation of the germ disc at the anterior end of the egg. Gastrulation and the establishment of multiple germ layers occur as the germ disc is condensing.

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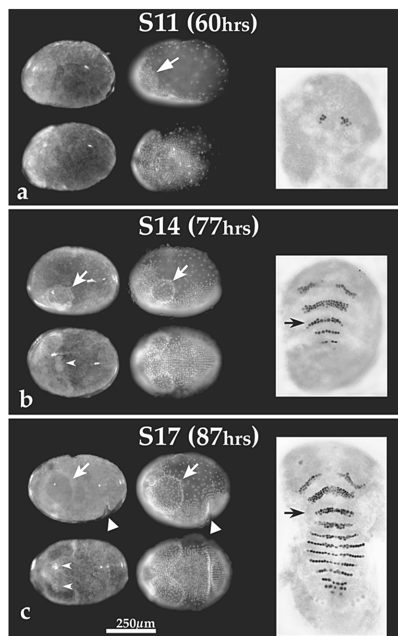

FIGURE 4. *P. hawaiensis* stages 11, 14, and 17 with Engrailed expression. Live bright-field and matching post-fixation DAPI images showing lateral (*upper* image pairs) and ventral (*lower* image pairs) views. (*Right*) Engrailed (En) expression for the corresponding stage, where all embryos are oriented anterior-up. Anti-En staining (with MAb 4D9) is black in these bright-field images. (Black arrows) The position of the mandibular (Mn) segment En stripe. (*a*) Stage 11 (S11); formation of ectodermal cell rows. (*Top* image pair, lateral view) The aggregation of cells forming the midgut anlagen; (white arrow) midgut anlagen nuclei in the DAPI field. (*Bottom* image pair, dorsal view) The organization of the ectoderm into transverse cell rows. First expression of En initiates in An1. (*b*) Stage 14 (S14); bilobed germ-cell cluster. (White arrow) Midgut anlagen becomes ovoid. (White arrowhead) Germ-cell clusters at the midline begin to separate from one another. Organization of the ectoderm into transverse cell rows is clearly visible in the DAPI image of the ventral view. En expression is throughout the cephalon, with the fifth stripe corresponding to the second maxillae segment (Mx2). (*c*) Stage 17 (S17); ventral flexure visible. (White triangles) Positioned where posterior ventral flexure initiates (T4/T5). (White arrows) Indicate the position of the expanding midgut anlagen. (White arrowheads) Flanking the midline in the (*lower*) ventral view indicate position of the migrating germ-cell clusters. Ventral flexure is clearly visible in the DAPI images. En expression is in 12 stripes; the 12th stripe corresponds to T7.

positions of both anterior/posterior and dorsal/ventral axes relative to the egg axis. Patterning of the embryonic ectoderm posterior to the head results from the condensation of a grid-like array of cells, the parasegment precursor rows (PSPR), which subsequently undergo two rounds of wave-like, mediolateral, mitotic division to generate four transverse rows of cells that constitute a parasegment. Further divisions, although reproducible, occur in much more complex patterns and begin to define primordia of ectodermal derivatives such as appendages and the central nervous system. Unlike the ectoderm, the underlying segmental mesoderm is generated via mesoteloblastic growth, as in other crustaceans. Mesoteloblasts pass through highly ordered series of asymmetric divisions to generate progeny called "mesoblasts." As they do so, the mesoteloblasts shift posteriorly beneath the developing ectodermal grid. The resulting mesoblasts are organized into rows beneath the posterior compartment of each of the forming parasegments, ultimately producing the somatic mesodermal derivatives of the associated segment. By stage 14 (77 h), the bilateral midgut anlagen have become two well-defined circular structures flanking segments T2 and T3. These will dramatically increase in size until they meet each other along their most ventral margins and begin to fuse. During development of the embryonic germ band, the germ cells become clearly visible underneath the developing maxillae-1 (Mx1) and maxillae-2 (Mx2) segments. Initially, they are present as a single medial cluster of large, whitish cells underneath the ectoderm. By stage 17 (87 h), the germ cells have separated into two bilaterally opposed, whitish clusters, which then begin to move laterally and posteriorly away from the embryonic midline. Anterior appendage primordia have begun to elongate, whereas thoracic appendages are visible as buds. As the germ band elongates, it begins to fold inward toward the egg interior at segment T5. This posterior (ventral) flexure then expands to include more anterior and posterior segments, appearing as a clear furrow in the germ band when viewed from the side.

Stages 19-30: Appendage Formation, Organogenesis, Neurogenesis, and Hatching

Some of stages 19-30 are depicted in Figure 5. The posterior (ventral) flexure becomes increasingly pronounced, broadening into a distinctive paddle-shaped structure by 96 h. By 112 h, the posterior paddle has significantly narrowed, limb buds are becoming apparent along the abdomen, and the developing telson is clearly visible and oriented toward the anterior. By stage 21 (120 h), all abdominal segments possess visible limb buds, and the hindgut proctodeum is just becoming visible at the posterior terminus. The developing midgut can be seen as an organized epithelial sheet spreading over the yolk. The germ-cell clusters are visible in the lower hemisphere of the midgut anlagen and are beginning to migrate dorsally in the embryo. By 132 h, the midgut completely envelops the remaining yolk, and thoracic limb buds have begun elongation. At 144 h, dorsal closure of the ectoderm over the gut appears to be nearly complete, and the digestive cecae first become apparent as

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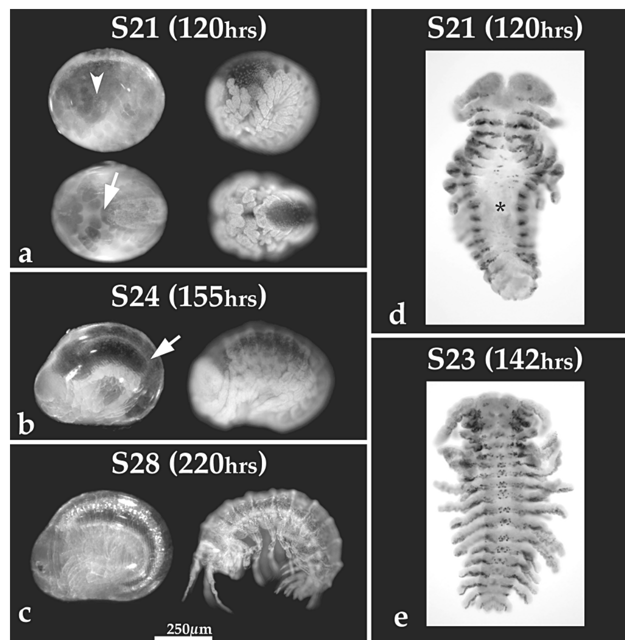

FIGURE 5. *P. hawaiensis* stages 21, 24, and 28 with Engrailed (en) expression. Live bright-field and matching post-fixation DAPI images showing (*upper* image pairs) lateral and (*lower* image pairs) ventral views in a and only lateral views in *b* and *c*. In *d* and *e*, the bright-field images of En expression are oriented such that anterior is up. (*a*) Stage 21 (S21); hindgut proctodeum becomes visible. (White arrowhead on lateral view, *upper*) Position of the germ-cell clusters. (*Lower*, ventral view, white arrow) The hindgut proctodeum is visible as a wedge-shaped ridge of cells at the posterior end of the embryo. (*b*) Stage 24 (S24); midpoint of digestive cecae extension. (White arrow) The posterior extent of extending cecum (~T7). Yolk granules have cleared from the cephalon. Body tergites and coxal plates are visible. (*c*) Stage 28 (S28); yellow cuticle. As the cuticle continues to accumulate, the embryo acquires a yellow-gold appearance not apparent in these black and white images. Eye rhabdomere fields would appear intensely red in color images, limbs have acquired their final morphology, and the digestive cecae are significantly depleted of yolk stores. (*d*) En expression, stage 21 (S21); all segments are En-positive. (Black asterisk) Indicates the prominent and transient ventral midline split. (*e*) En expression, stage 23 (S23); the ventral midline cleft has closed, and all segments possess a subset of En-positive neurons.

bud-like posterior projections from the now wedge-shaped midgut anlagen. Digestive cecae are major secretory organs consisting of a pair of blind tubes that open directly into the anterior end of the medial midgut. By stage 24 (155 h), the digestive cecae have extended approximately midway and are nearing the abdomen. By the end of digestive cecae extension (168 h), most yolk has relocated from the developing medial midgut to the digestive cecae. Eye fields become distinctly visible as two bilaterally symmetric, whitish clusters of rhabdomeres by 180 h. At this time, the tubular heart, located along the dorsal thoracic region, visibly begins to beat. At 192 h, pigment cells that surround the whitish rhabdomeres begin to develop a deep red color. By stage 28 (216 h), the embryo cuticle acquires a yellow-gold appearance. Beyond this point, detection of gene expression by in situ hybridization becomes problematic because the cuticle has thickened. The digestive cecae are significantly depleted of yolk stores, and the heart is beating strongly. Hatching occurs at ~250 h of development. Immediately before hatching, the embryo is in a constant state of coordinated motion. In addition, the embryo increases substantially in size immediately before and during hatching, most likely via active uptake of water. Soon after breaking free of the eggshell, hatchlings undergo a molt and revert to a smaller size.

GENETICS, GENOMICS, AND ASSOCIATED RESOURCES

The genome of *P. hawaiensis* is estimated to be 3.6 Gb in size (A. Aboobaker and N. Patel, unpubl.). The Joint Genome Institute (JGI) has undertaken both EST and directed BAC sequencing of *P. hawaiensis.* Both of these sequencing projects are nearing conclusion. In addition, the related amphipod crustacean *J. slatteryi*, whose genome size is 690 Mb, has been selected for fivefold genome sequencing, paired-end reads from 100,000 cDNA clones, and sixfold BAC end sequencing.

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EST Sequencing

Using a collection of *P. hawaiensis* embryos spanning the first two-thirds of embryogenesis (0-156 h), a normalized total embryonic cDNA library with an average insert size of 2.2 kb was constructed for EST sequencing; 29,566 cDNA clones were end-sequenced using the traditional methods of Sanger sequencing. Of the sequences passing quality control to cluster, 90% yielded a total of 47,732 unique ESTs with a mean read length of 670 bp. These ESTs were parsed into 13,366 clusters of highly related sequence. The resulting cluster distribution suggests that the source cDNA library successfully normalized gene coverage of the embryonic transcriptome: 68% of the total clusters contained sequence from only a single clone and only 66% of passing clones formed clusters with greater than 1 clone/cluster. A searchable web-based database of *P. hawaiensis* EST sequences is being developed at JGI, which will provide the scientific community ready access to these data.

To supplement the EST sequences, JGI will perform an additional single 454-FLX run on sheared cDNA to generate ~80 Mb of data in ~200-bp reads (400,000-600,000 clones). JGI will cluster the 454 ESTs with the ESTs already run on the Sanger platform.

Directed BAC Sequencing

A *P. hawaiensis* BAC library (F. Poulin and N. Patel, in collaboration with the C. Amemiya laboratory, unpubl.) was constructed from nuclear DNA isolated from a isofemale line "iso2" that was established ~6 yr ago. With an average insert size of 120-150 kb, the library was designed to cover the entire genome about five times. Because the size of the *Parhyale* genome is estimated to be 3.6 Gb, an array of 129,024 clones was plated onto seven high-density filters. Screens with single-copy genes confirmed fivefold to sevenfold coverage.

Probes made from cDNA sequences from about 60 developmentally important genes were used to screen the BAC library filters, using standard techniques for radioactive hybridization. Positive BAC clones for each probe were sized, screened by polymerase chain reaction (PCR), and restriction-mapped to ensure that the regions of interest were centered before being sent for sequencing. The Stanford Human Genome Center is in the process of sequencing 70 BAC clones.

TECHNICAL APPROACHES

Essential laboratory techniques for the study of embryogenesis in *P. hawaiensis* are described in **Fixation and Dissection of *Parhyale hawaiensis* Embryos** (Rehm et al. 2009a), **Injection of *Parhyale hawaiensis* Blastomeres with Fluorescently Labeled Tracers** (Rehm et al. 2009b), **Antibody Staining of *Parhyale hawaiensis* Embryos** (Rehm et al. 2009c), and **In Situ Hybridization of Labeled RNA Probes to Fixed *Parhyale hawaiensis* Embryos** (Rehm et al. 2009d).

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