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# The amphipod crustacean *Parhyale hawaiensis*: An emerging comparative model of arthropod development, evolution, and regeneration

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**Abstract**

Recent advances in genetic manipulation and genome sequencing have paved the way for a new generation of research organisms. The amphipod crustacean *Parhyale hawaiensis* is one such system. *Parhyale* are easy to rear and offer large broods of embryos amenable to injection, dissection, and live imaging. Foundational work has described *Parhyale* embryonic development, while advancements in genetic manipulation using CRISPR-Cas9 and other techniques, combined with genome and transcriptome sequencing, have enabled its use in studies of arthropod development, evolution, and regeneration. This study introduces *Parhyale* development and life history, a catalog of techniques and resources for *Parhyale* research, and two case studies illustrating its power as a comparative research system.

This article is categorized under:

Comparative Development and Evolution > Evolutionary Novelties
Adult Stem Cells, Tissue Renewal, and Regeneration > Regeneration
Comparative Development and Evolution > Model Systems
Comparative Development and Evolution > Body Plan Evolution

**KEYWORDS**

arthropod, crustacean, development, evodevo, evolution

## 1 | INTRODUCTION: CHOOSING A "MODEL" SYSTEM

Research organisms have long been essential to the study of genetics. Early animal systems, such as *Drosophila melanogaster* and *Mus musculus*, were used to uncover basic genetic principles through the study of spontaneous and induced mutant alleles (Cuenot, 2000a; Sturtevant, 1995). The ease of husbandry and short generation time of *Drosophila* in particular have enabled genetic screens that have revealed basic principles of animal development, such as axis formation, segmentation, body regionalization, cell fate specification, and tissue morphogenesis. As genetics became more specialized, researchers began to choose additional organisms for unique traits that made them ideal for studying particular topics. For example, the roundworm *Caenorhibitis elegans* was first established to study the development of the nervous system (Ankeny, 2003; Brenner, 2005).

Its simple behavior, short generation time, and hermaphroditic lifestyle allowed for genetic mutant analysis of traits otherwise difficult to study in other systems. Around the same time, the zebrafish *Danio rerio* was established to understand vertebrate genetics and development (Streisinger, Walker, Dower, Knauber, & Singer, 2003). Its small, transparent embryos proved useful for identifying developmental mutants.

New discoveries in each of these systems expanded the breadth of their research potential, while a number of technological breakthroughs greatly enhanced their usefulness and provided new ways to address long-standing biological questions. Examples include creating transgenic animals using transposable elements or homologous recombination, imaging molecular events in living animals using fluorescently labeled proteins, and carrying out reverse genetic screens using RNA interference (RNAi).

Work in such so-called "model" systems has uncovered basic principles of molecular, cell, and developmental biology. However, these classical systems are separated by vast phylogenetic distances and represent a tiny fraction of the planet's biological diversity. To understand the evolution of animals, it has become increasingly important for biologists to develop novel research systems. These systems are sometimes selected to bridge phylogenetic gaps between current systems, and enable investigations into the evolution of fundamental biological processes. In other cases, novel systems possess interesting properties not found in classical systems, and are thus able to reveal previously unknown biological processes. Finally, comparisons between novel and well-established systems can enable deeper understanding of animal development and diversity.

One of the outstanding questions in evolutionary biology is how animals have diversified over hundreds of millions of years. The most successful group of animals on the planet, measured by biomass (Bar-On, Phillips, & Milo, 1988a) and species diversity, is the arthropods, and their diversity has been the subject of scientific curiosity and investigation for centuries. Arthropods have many shared features, including their namesake jointed appendages (arthro means joint; pod means foot) and their hard exoskeletons, which they molt repeatedly over their lifetimes. Along with insects, other arthropod lineages include chelicerates (spiders, mites, and scorpions), myriapods (centipedes and millipedes), and non-insect crustaceans (copepods, crabs, barnacles, and many other organisms; Figure 1a). These organisms contain tremendous diversity in their development, body plan, and life history. However, much of the research on arthropod diversity has relied on a handful of genetically tractable research systems.

In the field of arthropod development, *D. melanogaster* remains the predominant research system. *Drosophila* has been the subject of many foundational studies, such as the discovery of the hierarchy of genes involved in segmentation (Nüsslein-Volhard & Wieschaus, 2002), body regionalization (Lewis, 2007), and embryonic axis formation (Driever and Nüsslein-Volhard, 1988a, 1988b), and many other developmental processes. *Drosophila* are easy and inexpensive to rear, tools for genetic manipulation are well established, and animal stock centers maintain numerous mutant lines for the large *Drosophila* research community. Over the years, various databases have been built to store genetic, genomic and bioinformatic data for *Drosophila*, such as Flybase.org. With the discovery of genome-editing technology such as CRISPR-Cas9, mutant and transgenic alleles are even easier to generate. Thus, *Drosophila* remains a key system for the study of many biological phenomena.

Though *Drosophila* offers many resources, it represents but one type of organism within the tremendous morphological diversity of arthropods. For example, *Drosophila*'s two-winged, six-legged body plan stands in contrast to other insects, which may have two pairs of wings, or no wings at all. Arthropods outside of the insects have even greater body plan diversity, with extreme examples in the myriapods, which can have over 100 pairs of limbs. Comparative studies have also demonstrated that some aspects of *Drosophila* development are derived relative to those of other arthropods. During embryogenesis, *Drosophila* undergoes long-germ development, wherein all body segments form simultaneously towards the end of a syncytial stage (Davis & Patel, 2002). In contrast, most insects undergo short-germ or intermediate-germ development, wherein only anterior segments are specified during the early syncytial stage, while more posterior segments are successively established in a cellular environment; in contrast, other arthropod groups lack a syncytial stage entirely (Davis & Patel, 2002). Moreover, some key *Drosophila* segmentation genes, such as the homeobox-containing genes *fushi tarazu*, *even-skipped*, *zen*, and *bicoid*, have changed dramatically in expression and function when compared to homologs in species within early-branching arthropod lineages (Hughes & Kaufman, 2010). Thus, while *Drosophila* has provided an excellent platform for studying general developmental principles, some aspects of its development are not representative of more general, and apparently ancestral, arthropod development.

Other models have expanded upon the foundational work in *Drosophila* to inform our understanding of arthropod development and evolution. For example, the flour beetle *Tribolium castaneum* has been offered as a more representative insect research system. Its intermediate-germ development and leg-bearing larval stages are features shared with a broader diversity of insect clades (Davis & Patel, 2002; Lynch, El-Sherif, & Brown, 2017). *Tribolium* has been used as a comparative model to examine how pair-rule genes (Brown, Hilgenfeld, & Denell, 1999a; Choe, Miller, & Brown, 1999b; El-Sherif, Averof, & Brown, 2000b; Sarrazin, Peel, & Averof, 2016) *Hox* genes (Beeman, 2011; Tomoyasu, Wheeler, & Denell, 2001), and other

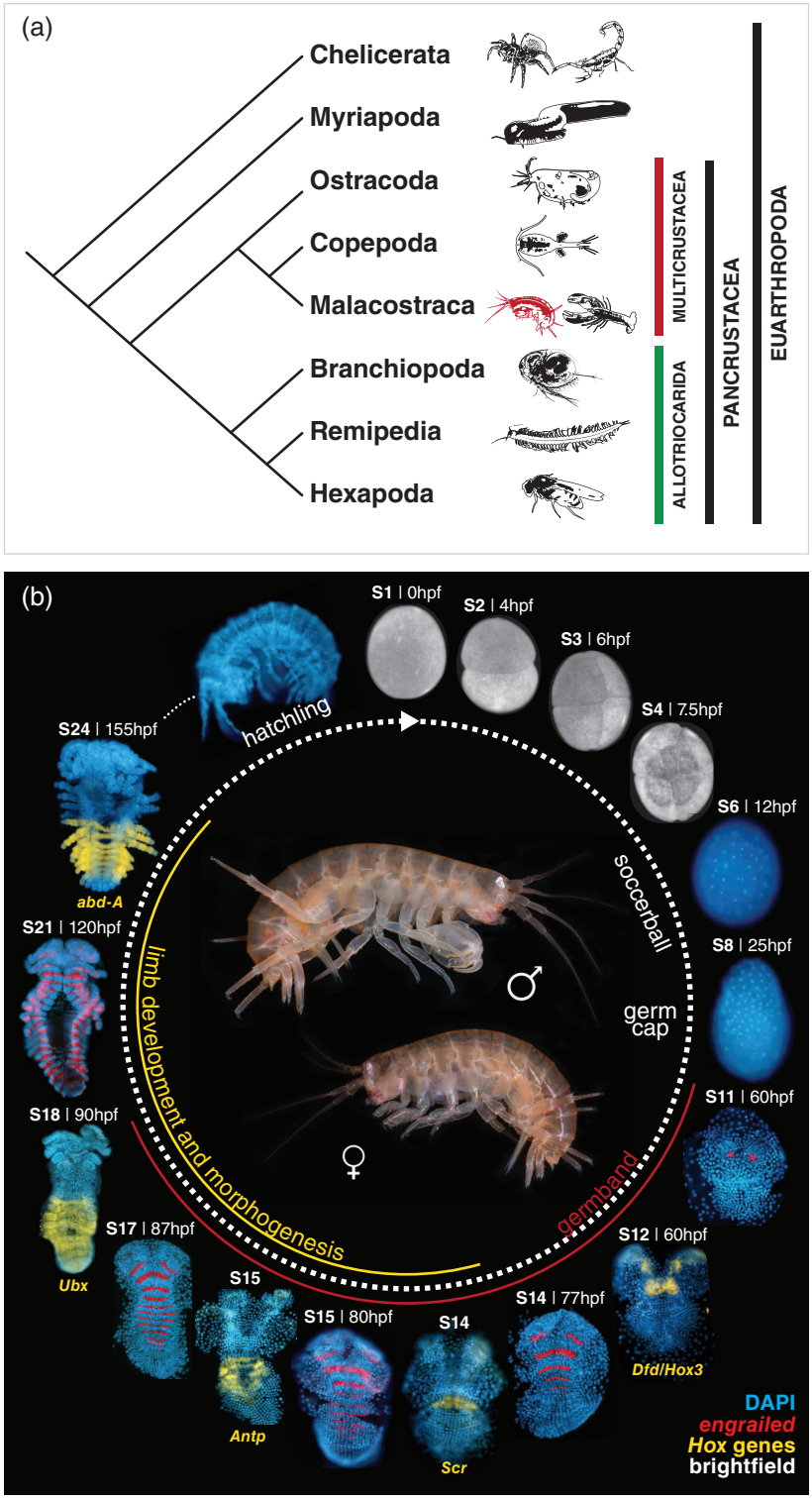


**FIGURE 1** Phylogeny and development of *Parhyale hawaiensis.* (a) *P. hawaiensis* is a malacostracan crustacean, and is within a clade with lobsters, crabs, and shrimp. Crustaceans are one of the four major groups of arthropods. The three remaining groups are chelicerates, including spiders and scorpions; myriapods, including millipedes and centipedes; and hexapods, including insects. (Schwentner, Combosch, Pakes Nelson, & Giribet, 1988b). (b) *Parhyale* development takes place over ~10 days. The early embryo undergoes rounds of holoblastic cleavage (Stage 1–Stage 4) and cells eventually coalesce into a germ cap (Stage 8). Much of the embryo develops on the surface of the yolk during germband elongation. Parasegment formation can be visualized by the expression of *engrailed* (red). Over the course of embryogenesis, the *Parhyale Hox* genes (yellow) initiate expression over different regions of the body in a collinear anterior–posterior temporal sequence. The hatchling emerges as a miniature version of the adult. Sexually mature adults display sexual dimorphism: females are smaller and have visible ovaries, while males are larger and have larger chelipeds on T3. hpf, hours post-fertilization; *Dfd*, deformed; *Scr*, sex combs reduced; *Antp*, antennapedia; *Ubx*, ultrabithorax; *AbdA*, abdominal-A

developmental programs that have evolved within *Insecta*, particularly by comparing its developmental processes to those of *Drosophila.* Such studies have provided mechanistic insight into how genes and developmental processes change over evolutionary time. In addition to gene expression data, genetic manipulations in *Tribolium*, such as parental RNAi (Bucher, Scholten, & Klingler, 1997), have provided methods to efficiently probe gene function, and even carry out forward genetic screens (Christian et al., 1965; Knorr, Bingsohn, Kanost, & Vilcinskas, 2018). The development of such techniques has made *Tribolium* an appealing system for testing hypotheses about the course of evolution at long timescales. Beyond insects, gene expression data and some functional data from crustaceans, chelicerates, myriapods, and onychophorans have provided

insights into the evolution of body plan diversity within the arthropods and other closely related clades (Hughes & Kaufman, 2010; Janssen, Eriksson, Tait, & Budd, 1987; Pace, Grbić, & Nagy, 2012; Paese, Schoenauer, Leite, Russell, & McGregor, 1974; Schwager, Pechmann, Feitosa, McGregor, & Damen, 1994; Schwager, Schoppmeier, Pechmann, & Damen, 2005). By synthesizing the observations made in these disparate systems, evolutionary biologists can develop a more comprehensive understanding of the genetic and developmental processes that lead to organismal diversification.

To enable broader comparative studies of arthropod development, Browne, Price, Gerberding, and Patel (2006) established the amphipod crustacean *Parhyale hawaiensis* (Dana, 2002) as a novel research system (Gerberding, Browne, & Patel, 2012). *P. hawaiensis* (hereafter referred to in short as *Parhyale*) is a phylogenetically strategic choice that serves as an outgroup for insects (Oakley, Wolfe, Lindgren, & Zaharoff, 2015; Schwentner et al., 1988b). As a crustacean, it serves as a representative member from an ancient group of organisms that traces its origin to the Cambrian era (Schwentner et al., 1988b), and which have remained tremendously successful in diversification since their emergence. As a malacostracan crustacean, it is also closely related to commercially important shrimp, lobsters, and crabs. Moreover, its life history makes it amenable to laboratory culture and molecular genetic manipulation. *Parhyale* are shallow-water, circumtropical detritivores with a high tolerance for environmental osmotic changes (Barnard, 2006; Poovachiranon, Boto, & Duke, 2015; Shoemaker, 2017). Their large embryos are easy to collect and image; though the yolk is opaque, the superficially positioned embryo and surrounding chorion are transparent, and a thorough staging scheme enables researchers to identify the relative developmental stage of any embryo (Browne et al., 2006). In contrast to many other crustaceans, which emerge as planktonic nauplii larvae that subsequently metamorphose into adults and can thus be difficult to culture, *Parhyale* hatchlings emerge as miniature versions of adults, and can easily be raised to adulthood to establish new genetic lines. These features have made *Parhyale* an ideal candidate to become a new research system, and indeed, continued technique development over the last two decades has advanced its tractability. Published protocols describe techniques for embryo injection (Rehm, Hannibal, Chaw, Vargas-Vila, & Patel, 1905), dissection (Rehm, Hannibal, Chaw, Vargas-Vila, & Patel, 1853), antibody staining (Rehm, Hannibal, Chaw, Vargas-Vila, & Patel, 2005), and in situ hybridization (Rehm, Hannibal, Chaw, Vargas-Vila, & Patel, 2002), enabling new researchers to explore *Parhyale* with ease.

An explosion in nucleotide sequencing technologies has facilitated gene discovery in *Parhyale*, while advances in genetic manipulation have enabled studies of gene function. Such technological advancements have enabled *Parhyale* researchers to examine several aspects of *Parhyale* development and address some important issues in the evolution of arthropods. Initial genetic approaches used the *Minos* transposase system to generate transgenic animals for misexpression (Pavlopoulos et al., 2014), while morpholinos, injected siRNA, and expressed RNA hairpins have enabled gene knockdown (Liubicich et al., 2012; Martin et al., 2005; Özhan-Kizil, Havemann, & Gerberding, 2017). Since the start of the genome-editing revolution, CRISPR-Cas9 programmable nucleases have been used to generate knock-out and knock-in animals (Kao et al., 2002; Martin et al., 2005; Serano et al., 2004). Meanwhile, early genomics efforts included the isolation of conserved genes using polymerase chain reaction (PCR) and rapid amplification of cDNA ends (RACE), as well as the generation of a bacterial artificial chromosome (BAC) library, from which early insights into genome structure were achieved (Parchem, Poulin, Stuart, Amemiya, & Patel, 2013). As sequencing costs have plummeted, several different groups have published transcriptome sequencing data of both the adult and embryonic animals (Blythe et al., 2012; Nestorov, Battke, Levesque, & Gerberding, 2014; Zeng et al., 2002; Zeng & Extavour, 2014). A recent study describes the *Parhyale* genome, positioning *Parhyale* for a new age of genome-scale investigations (Kao et al., 2002). Moreover, an active and expanding group of *Parhyale* researchers has begun to curate protocols and other resources to facilitate the expansion of the community (see *Parhyale* Community Website: http://parhyale.rc.fas.harvard.edu/).

Since its introduction as a research system almost two decades ago, *Parhyale* has enabled investigations that address key questions in developmental and evolutionary biology. For example, its short-germ development has allowed investigation into mechanisms of embryonic axis formation and segmentation in the absence of a *Drosophila*-like embryonic syncytium (Browne et al., 2006; Extavour, 2012; Gupta & Extavour, 2016; Nestorov et al., 2014; Özhan-Kizil et al., 2017; Vargas-Vila, Hannibal, Parchem, Liu, & Patel, 2013). These studies have offered clues to the ancestral function of genes critical to dorsal–ventral patterning and neural specification in arthropods. In another set of studies, researchers discovered correlations between germ layer identity and the differential distribution of mRNAs and other cytoplasmic components at the 8-cell stage of development (Extavour, 2012; Gupta & Extavour, 2016; Nestorov et al., 2014; Özhan-Kizil et al., 2017). *Parhyale* has also been used to explore how germ layers communicate during development, and how the embryo compensates within germ layers to recover lost tissue (Hannibal, Price, & Patel, 2016; Price, Modrell, Hannibal, & Patel, 2014).

In this review, we summarize much of the work that has been accomplished to date. We begin with a comprehensive summary of *Parhyale* life history and embryonic development, and then describe techniques for genetic manipulation and

resources for genomic analysis. Finally, we describe two case studies that illustrate the power of *Parhyale* as a comparative system for studying body plan evolution and regeneration. We review how work in *Parhyale* has provided insights into the evolution of arthropod body plan diversity, through studies examining the expression and function of *Hox* genes in this outgroup for *Drosophila* and all other insects. Then, we discuss how a striking discovery about the mechanisms of *Parhyale* limb regeneration mirrors findings from vertebrate systems, and how the optical properties of this small crustacean make it an ideal platform for future regenerative studies.

## 2 | DEVELOPMENT, ANATOMY, AND LIFE CYCLE

Within *Crustacea*, *Parhyale* belong to the class *Malacostraca* (Figure 1a). The Malacostracan clade is composed of familiar shrimp, krill, crabs, and lobsters, in addition to crustaceans with other unique body forms. *Amphipoda*, the order to which *Parhyale* belongs, are commonly referred to as scuds, side swimmers, or beach hoppers, and are a ubiquitous sight on beaches. *Parhyale* are detritivores with a circumtropical, intertidal, and shallow-water marine distribution (Shoemaker, 2017; Barnard, 2006). The animals are easy and inexpensive to rear and reproduce year-round, making them ideal for laboratory work.

*Parhyale* embryos can be collected from the ventral brood pouch of adult female animals without harm to either the mother or the embryos. Adult females can grow up to 20 mm in length, and can produce large broods of embryos (50+). Development has been thoroughly characterized into a series of morphologically identifiable stages (Browne et al., 2006; Figure 1b), and is consistent with observations in other amphipod crustaceans, such as *Orchestia cavimana* (Wolff & Scholtz, 2011). Embryonic development lasts about 11 days, beginning with a mediolateral holoblastic (total) cleavage at 4 hr that generates a 2-cell embryo (Figure 2a). After two additional stereotyped divisions, the embryo at the 8-cell stage contains four micromeres (small cells, abbreviated in lowercase) and four macromeres (large cells, abbreviated in sentence case; Figure 2b). At 12 hr, the embryo contains ~100 cells of about equal size. This is termed the "soccerball" stage for its appearance. Shortly after, cell cytoplasm separates from yolk, and cells migrate along the surface of the embryo into two predominant clusters of cells, one at the presumptive ventral end of the embryo, which will produce the ectoderm anlagen, and the other into a small circular cluster of visceral mesoderm and germ cells, referred to as the "rosette" (Figure 2b). After this stage, the majority of *Parhyale* morphogenesis remains at the surface of the embryo, allowing for easy live imaging of development.

Gastrulation occurs at around 20 hr, when the rosette migrates beneath the ectoderm anlagen (Alwes, Hinchen, & Extavour, 2009; Browne et al., 2006; Chaw & Patel, 1978; Figure 2c). This generates the "germ disc" stage. Germ disc cells then migrate and proliferate in the transition to the "germband" stage (Browne et al., 2006; Figure 2d,e). During the initial phase of germband elongation, cells of the posterior ectoderm organize into transverse rows of cuboidal cells referred to as "parasegment precursor rows" (PSPRs), which together create a grid-like germband (Figure 2f). The first of these cells to become morphologically distinct will align along the midline of the embryo and express *orthodentical* (Browne, Schmid, Wimmer, & Martindale, 2009). These midline precursor cells will later express *single-minded*, which is critical for specifying the dorsal–ventra axis of the *Parhyale* embryo (Vargas-Vila et al., 2013). Two waves of mitotic division sweep across the PSPRs, beginning adjacent to the midline and spreading laterally, generate two cells (a/b and c/d), then four cells (a, b, c, and d) from each original row of cells. These waves of division are highly organized; more anterior PSPRs form first and begin dividing as more posterior PSPRs are beginning to organize. The anterior-most cell row (a) expresses *engrailed*; each original PSPR row is thus equivalent to a parasegment in *Drosophila* (Figure 2h). The sequential addition of *engrailed* stripes provides a benchmark for staging *Parhyale* embryos (Browne et al., 2006).

A well-documented feature of *Parhyale* development is the presence of precise lineage restriction, particularly between ectoderm and mesoderm (Gerberding et al., 2012; Price et al., 2014). Lineage tracing has revealed that *Parhyale* restricts germ layers at the 8-cell stage (Figure 2b). Two of the macromeres, "El" and "Er", produce the anterior left and anterior right ectoderm, respectively, while the macromere "Ep" produces the posterior ectoderm and the midline of the germband. During germband elongation, there is some mixing between descendants of "El" and "Er" in the head segments, but the gnathal and thoracic segments appear to have a distinct left–right division. Additionally, the boundary between "El"/"Er" contribution and "Ep" contribution to the posterior ectoderm appears to be determined stochastically.

Cell ablation experiments have demonstrated that "El," "Er," and "Ep" represent an "ectoderm group" that is able to undergo intra-germ layer compensation as late as gastrulation, but not after initial germband formation (Price et al., 2014; Figure 3a). "Ep" lineage cells, when ablated, can be replaced by "El" and "Er" cells together, while "Ep" cells can also replace either an ablated "El" or "Er." Notably, "El" and "Er" cells retain their left/right distinctions when compensating for the ablated "Ep" cell.

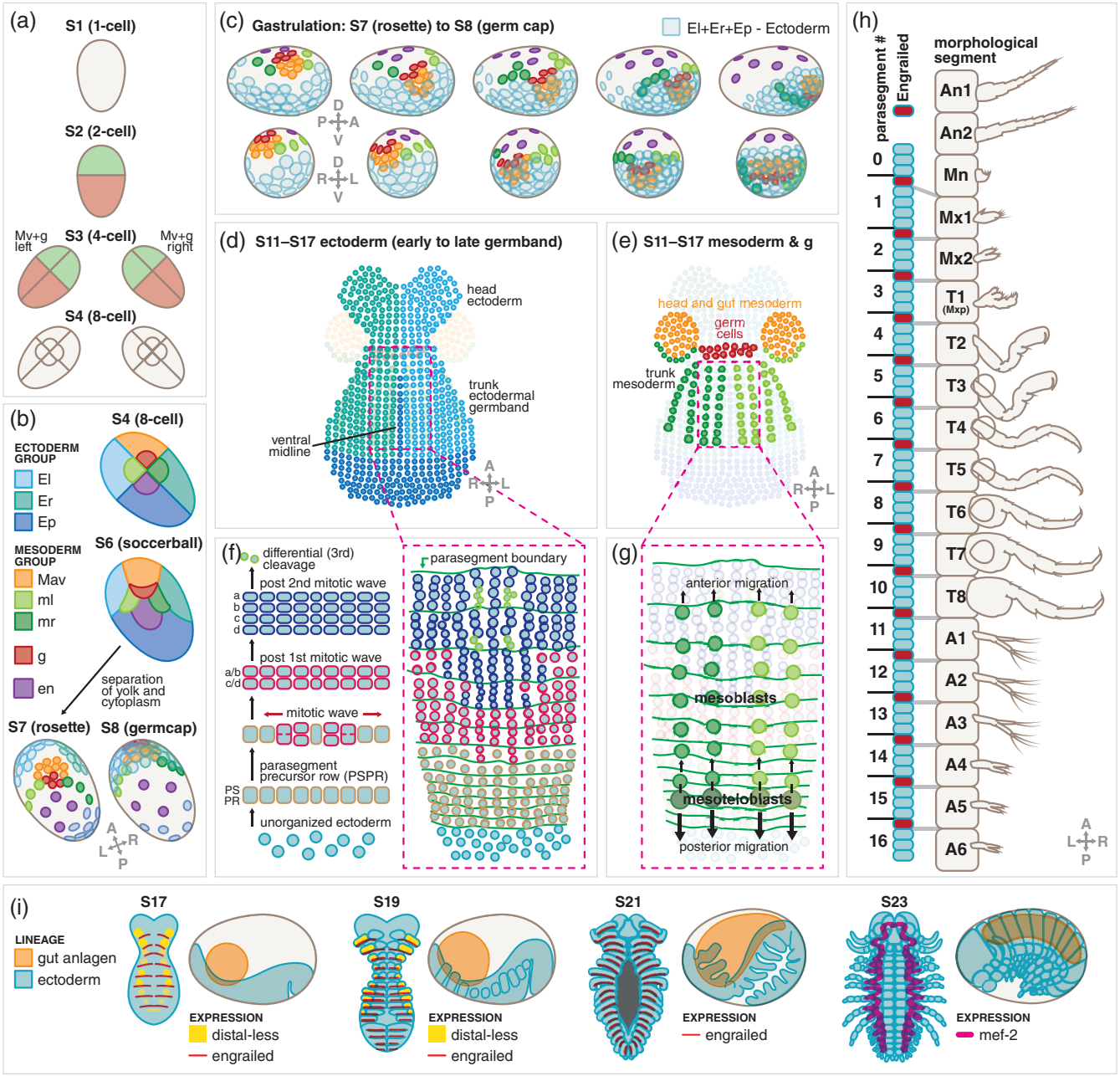


**FIGURE 2** Parhyale cell lineage and development. (a) Following fertilization, the embryo undergoes a series of stereotyped holoblastic cleavages. At the 4-cell stage, the embryo can either undergo left or right cleavage, characterized by the position of the Mv+g cell. (b) Lineage tracing has revealed that descendants of cells of the eight-cell stage display germ layer restriction. The eight-cell embryo contains four macromeres (El, Er, Ep, Mav) and four micromeres (ml, mr, en, g). During the transition from S6 to S7, cells extrude their yolk to separate the cytoplasm from the yolk. The cells in the rosette of the S7 stage migrate beneath a layer of ectodermal cells in a process associated with gastrulation to generate the S8 germ cap stage. (c) Illustration of cell migrations during gastrulation. (d) Illustration of the contributions of El, Er, and Ep cells to the ectoderm at the germband stage. (e) Illustration of the contributions of ml, mr, and Mav cells to the mesoderm and the position of the germ cells at the germband stage. (f) Left, schematic diagram of the cell division cycles that give rise to the embryonic parasegments. Unorganized cells in the posterior of the embryo organize to form PSPRs, which then divide once to generate a/b and c/d cells, then again to generate a, b, c, and d cells. These divisions occur as mediolateral waves of division, beginning with the cells immediately next to the midline, and then spreading laterally. The midline cells divide shortly after the cells that are adjacent to them. Right, illustration of the organization of cells at the germband stage. (g) Migrations of the mesoteloblasts and mesoblasts during germband elongation. Illustration represents a subset of the mesoteloblasts. Mesoteloblasts migrate posteriorly and divide to deposit mesoblasts. Mesoblasts then migrate anteriorly to pair with their specified ectodermal parasegments. (h) Trunk ectodermal parasegments and their corresponding out-of-phase morphological segments. The "a" cell in each parasegment expresses *engrailed*, which marks the posterior of each morphological segment. (i) Gene expression markers and morphological markers in *Parhyale* development. At S17-S19, *engrailed* marks the anterior of each parasegment, while *distalless* marks the developing limb buds. At S21, *engrailed* marks the parasegments, and at S23, *mef-2* marks the developing mesoderm. The gut anlagen, which sequesters the yolk and develops into the gut, is a prominent feature of embryos, and is useful in classifying embryonic stages. A, anterior; P, posterior; D, dorsal; V, ventral; L, left; R, right; An, antenna; Mn, mandible; Mx1, maxillule; Mx2, maxilla; Mxp, maxilliped


**FIGURE 3** Germ layer compensation and germ layer interactions in *Parhyale*. For ease of comprehension, in this figure, embryos are illustrated with the dorsal side facing towards the reader. During actual development, the dorsal side of the embryo faces the yolk, while the ventral side is superficial and clearly visible. (a) Left column: illustration of wild-type lineage patterns and morphology for ectoderm-lineage blastomeres. Middle and right columns: illustrations of ablation experiments, demonstrating intra-germ layer compensation in the ectoderm. Ablation of any two cells from the ectoderm lineage results in embryonic lethality. (b) Ablation of ectoderm lineage cells after the start of germband elongation. Germ layer compensation does not occur after the start of the germband stage. (c) Left column: illustration of wild-type lineage patterns and morphology for mesoderm-lineage blastomeres. Middle and right columns: illustrations of ablation experiments demonstrating intra-germ layer compensation in the mesoderm. (d) Ablation of mesoderm lineage cells after the start of germband elongation. Germ layer compensation does not occur after the start of the germband stage. (e) Ablation of the mesoderm after the start of germband elongation does not affect ectodermal segmentation. (f) Ablation of the ectoderm after the start of germband elongation leads to defects in mesoblast division and migration

Two micromeres and one macromere, "ml," "mr," and "Mav," together produce the mesoderm, including the head mesoderm, the visceral mesoderm, and a set of teloblasts that produce the segmented mesoderm of the body (Gerberding et al., 2012; Price et al., 2014). The anterior mesoderm, primarily composed of "Mav" descendants with posterior contribution from

"ml" and "mr," forms two circular populations of cells at the germband stage that eventually migrate ventrally to form the head and visceral mesoderm (Figure 2e). The posterior teloblastic mesoderm, which arises from "ml" and "mr" descendants, produces the left and right mesoderm, respectively (Figure 2e). Lineage mixing between "ml" and "mr" descendants is rare, but occasionally observed. During germband elongation, eight mesodermal stem cells (four on each side of the embryo; descended from mr and ml; called mesoteloblasts) migrate posteriorly, depositing rows of daughter cells, termed mesoblasts, beneath the ectoderm (Gerberding et al., 2012; Figure 2g). (For a detailed analysis of the early mesoteloblast divisions, see Price & Patel, 2012.) The mesoblast rows eventually align with each ectodermal parasegment, and go on to divide and differentiate into the muscle cells of each body segment (Price & Patel, 2012).

Cell ablation experiments have also demonstrated that the "ml," "mr," and "Mav" cells represent a "mesoderm group" (Price et al., 2014; Figure 3d). Ablation of any one of the cells can be compensated for by other members of the group. As with the ectoderm group, "ml" and "mr" compensate for an ablated "Mav." Moreover, ablation of two of the cells in the mesoderm group can be compensated for entirely by the single remaining cell. Ablating all three mesoderm group cells results in a mesoderm-free embryo indicating that there is no inter-layer compensation between mesoderm and ectoderm lineages (Price et al., 2014).

Additional ablation experiments have demonstrated that proper mesodermal segmentation is dependent on signals from the ectoderm, while the ectoderm is capable of segmentation and morphogenesis independent of mesoderm (Hannibal et al., 2016). Ablation of all three mesoderm group cells results in an embryo with properly patterned ectoderm, but no muscle tissue, as visualized by *Mef-2* in situ hybridization. Meanwhile, ablation of either the El or Er cell after the initiation of germband elongation, after which intra-germ layer compensation no longer occurs, causes defects in mesoteloblast division and migration (Hannibal et al., 2016).

The micromere that sits above the smallest macromere is named "g" and gives rise to the germline (Gerberding et al., 2012). This cell inherits a specialized germ plasm that is sequestered as early as the 1-cell stage and is visible in living embryos (Gupta & Extavour, 2016). During early germband elongation, "g" descendants sit in a single cluster beneath the ectodermal row that will give rise to the future mandible. This cluster divides and migrates in two separate clusters until each eventually settles in its final location on the dorsal side of the hatchling.

After limb development and organogenesis, animals hatch as miniature versions of adults (Browne et al., 2006). The body plan of *Parhyale* contains 20 segments, 19 of which have appendages. There are six head segments: an ocular segment (which does not contain an appendage), two antennal segments (An1, An2), and three segments with feeding appendages: mandible (Mn), maxillule (Mx1), and maxilla (Mx2). The thorax consists of eight segments, beginning with a segment bearing a feeding-like appendage called a maxilliped (T1/Mxp). T2 and T3 segments have chelipeds, or claws; in sexually mature males, the T3 cheliped is enlarged and used to grasp onto females before copulation. T4–T8 segments have pereopods, or locomotary legs; T4–T5 legs face forward and are used for walking, while T6–T8 legs are oriented in opposition to T4–T5, and are used when the animal backs up or "jumps" rapidly. Finally, the abdomen consists of six segments with biramous appendages, with A1–A3 segments bearing pleopods, or swimming appendages, and A4–A6 bearing uropods, or anchoring appendages.

About 2 months after hatching, animals become sexually mature. Adults are sexually dimorphic: females are smaller and have prominent ovaries, which are visible through the body wall, while males are larger, and have a pair of larger chelipeds on T3. Animals ready to mate can be found in pairs, with males clasping females before copulation and releasing females after sperm has been deposited. This pre-copulatory *amplexus* allows for simple identification of animals ready to produce new embryos, allowing for easy collection of early embryos for injections to perform genetic manipulation and analysis.

## 3 | TOOLS FOR GENETIC MANIPULATION

To investigate gene function in any research organism, it is critical to have tools to manipulate gene activity, as well as to generate mutant or transgenic alleles. In the field of evolutionary developmental biology, researchers are especially dependent on technology development, and often make use of techniques first developed in other research organisms. Over the past decade, techniques for gene knockdown, knockout, and transgenesis have been adapted for *Parhyale.* These techniques take advantage of the ease of collecting and injecting early-stage embryos, and the long time window of the first several cell divisions.

Transgenesis in *Parhyale* was first achieved using the *Minos* transposon from *Drosophila hydei* (Pavlopoulos & Averof, 2015). This transposon has been used for transgenesis in a variety of animal systems, and consists of two terminal inverted repeats flanking a desired insert sequence (example in Figure 4a). Transgenesis can be achieved by injecting a recombinant construct containing the inverted repeat sequences along with *Minos* transposase mRNA into a 1- or 2-cell *Parhyale* embryo. The *Minos* construct will be randomly inserted into the genome, integrating transgenic alleles in up to 30% of surviving

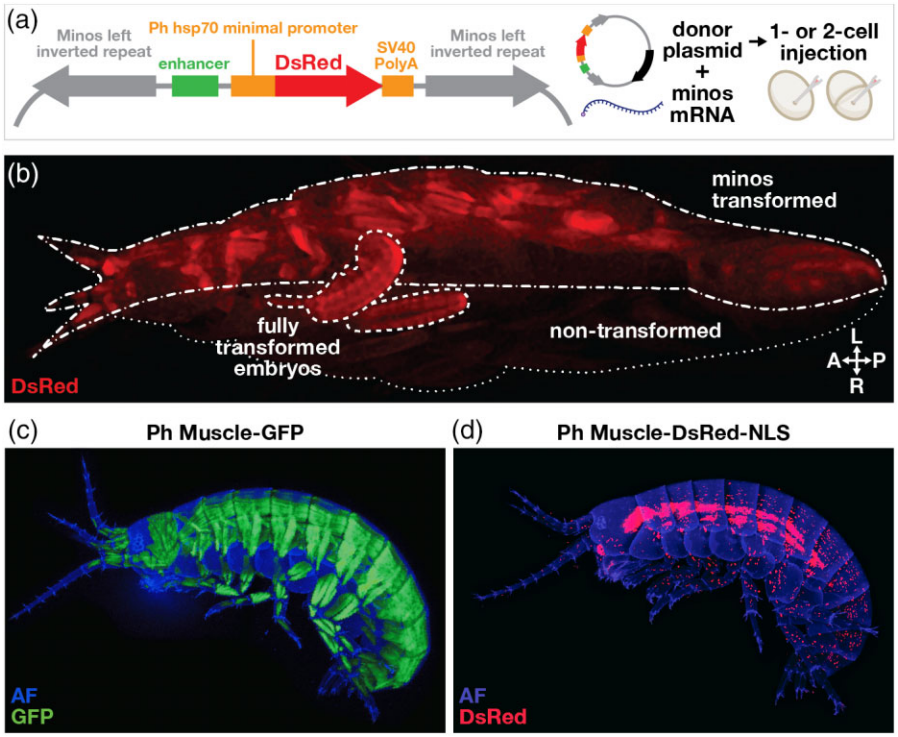


**FIGURE 4** Transgenesis in *Parhyale* using the *Minos* transposase system. (a) A basic *Minos* transposase donor plasmid and a schematic diagram of the transgenesis experiment. (b) Fluorescence image of a *Minos*-transformed female *Parhyale* that exhibits transformation in one-half of the body, with fully transgenic hatchlings in the brood pouch. (c) An example of transgenic animal in which *GFP* expression is specified by a *Parhyale* muscle enhancer. (d) Same as in c, but using *DsRed* that contains a nuclear localization signal (NLS). The bright signal that runs the length of the body is autofluorescence

hatchlings (Pavlopoulos & Averof, 2015). *Minos* was first tested by generating animals that expressed DsRed in cells around the eye using a synthetic 3xP3 enhancer, and was subsequently used to identify a muscle-specific enhancer upstream of the *Parhyale hsp70* protein. This approach has allowed for the generation of many transgenic lines (examples in Figure 4b,c; Pavlopoulos & Averof, 2015).

More sophisticated transgenic approaches have also been developed for *Parhyale*. Temporal control of gene expression was first achieved in *Parhyale* through the identification of a heat shock element from the *Parhyale hsp70* gene (Pavlopoulos et al., 2014). Placing transgenic animals bearing the *hsp70* heat shock element at 37°C for 30 min is sufficient to transiently induce high levels of transgene expression within a few hours. This technique has been used to misexpress *Ubx* in mosaic animals to study the effect of this *Hox* gene on segment identity across the *Parhyale* body plan (Pavlopoulos et al., 2014). Another method, called "integrase-mediated trap conversion" for gene trapping and trap conversion in *Parhyale*, used the *Minos* system to deliver a splice acceptor isolated from upstream of the *Hsp70* gene to a random genomic location (Kontarakis et al., 2013). *Minos* donor plasmid containing *Hsp70a*-DsRed or enhanced green fluorescent protein was injected into 1-cell embryos to generate a diverse catalog of different transgenic lines, in which each established line labels a different portion of the animal, depending on available splice donor exons from genes in the neighborhood of the *Minos* insertion. Another feature of this construct is the presence of an attB or attP sequence, which allows for insertion of additional alleles or the replacement of alleles using the phi-C31 integrase system (Groth, Fish, Nusse, & Calos, 1980). Using such transgenic tools, one can label and clone developmental and tissue-specific genes.

Genetic knockdown techniques in *Parhyale* have been established using morpholino and RNAi technology. Morpholinos have been used to knockdown the *vasa* gene, which is a well-studied germ cell determinant conserved across many arthropods (Özhan-Kizil et al., 2017). This experiment suggested that *Parhyale vasa* is not necessary for the establishment of the "g" cell, but it is critical for the maintenance of germline identity. At about the same time, small interfering RNAs (siRNAs) were used to knockdown the leg patterning gene *Distalless* and the *Hox* gene *Ultrabithorax* (Liubicich et al., 2012), producing a loss of distal limb elements and a transformation of the T2 and T3 segment towards a more anterior T1 fate, respectively. In addition to siRNA, engineered RNA hairpins have also been used to achieve gene knockdown via RNAi. By expressing a heat-shock-controlled *abd-A* sense/antisense fusion transcript linked with a *Drosophila white* intron, Martin et al. (2005) were able to generate knockdown

animals that helped uncover the function of the *abd-A* gene. These technologies often produce a range of phenotypes due to variation in the extent of knockdown, which can be useful for understanding how gene dosage contributes to specific phenotypes.

The field of genome engineering has recently been revolutionized by the discovery of the CRISPR-Cas9 programmable nuclease system (Doudna & Charpentier, 2013; Hsu, Lander, & Zhang, 2009; Jinek et al., 2016). Following double-stranded cleavage at a target site specified by a guide RNA, native DNA repair machinery can either induce indel mutations, or can be

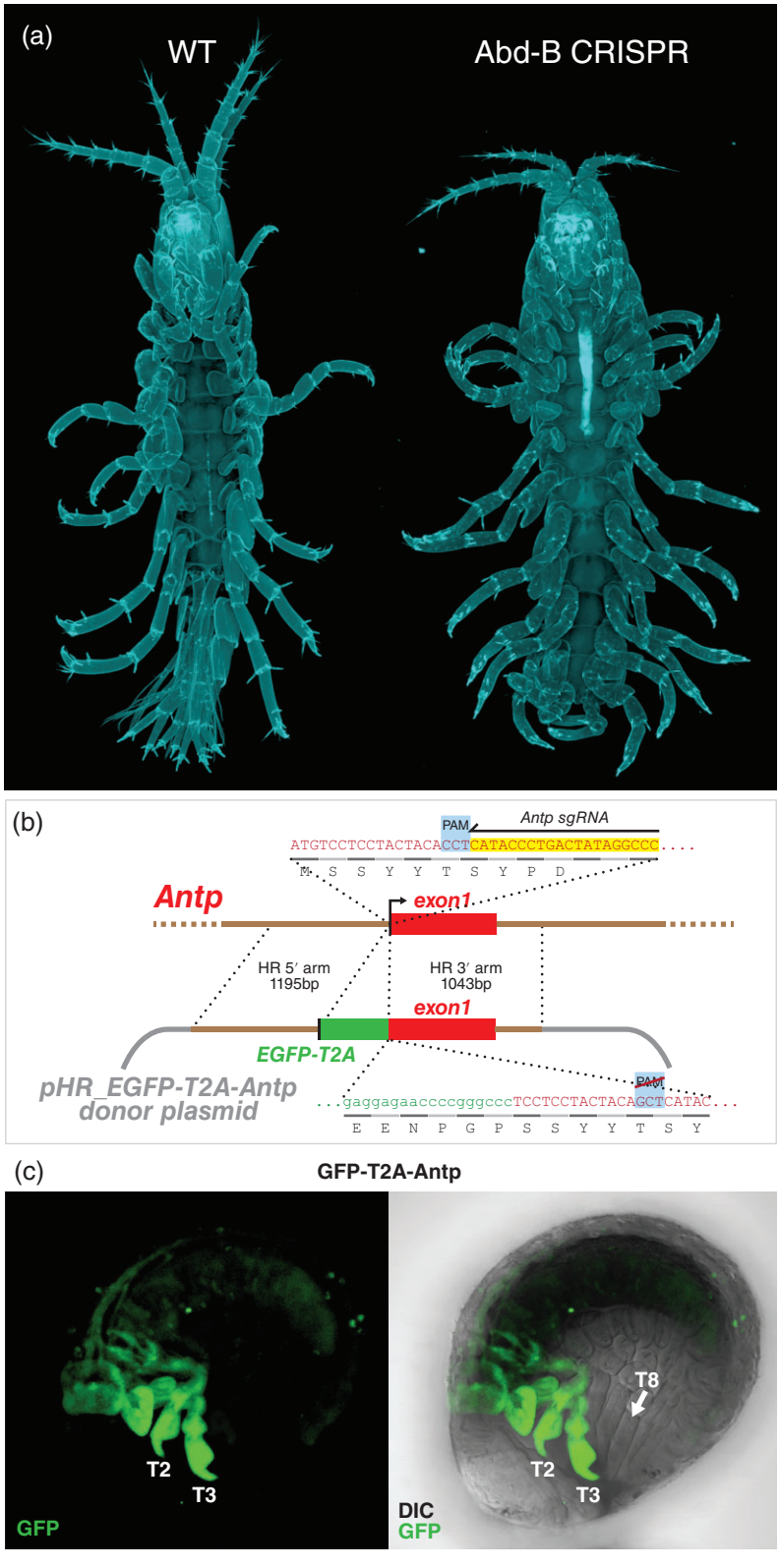


**FIGURE 5** CRISPR-Cas9 mutagenesis and transgenesis in *Parhyale*. (a) Bilateral CRISPR-Cas9 knockout of Abd-B. Left panel shows a wild-type *Parhyale* hatching. Right panel shows a CRISPR-Cas9 mutant for *Abd-B*. The abdominal appendages in the mutant are transformed to thoracic walking and jumping leg identities. (b) CRISPR-Cas9 homologous recombination plasmid and strategy to insert GFP into the *Antp* locus. (c) CRISPR-Cas9 homologous combination-mediated transgenesis. Transgenic embryo wherein GFP is expressed in segments that express Antp (Reprinted with permission from Martin et al. (2015). Copyright 2016 Elsevier Ltd.)

coopted to introduce exogenous DNA through homologous recombination, among many more advanced techniques. This transformative technology has enabled researchers of many organisms to perform genome editing and other genetic manipulations.

In *Parhyale*, the CRISPR-Cas9 system was first used to systematically knockout a large repertoire of *Hox* genes in order to examine their function in specifying segment and appendage identity in G0 embryos (Martin et al., 2005; example in Figure 5a). It was also used for homologous recombination with double-stranded DNA plasmids to generate animals expressing EGFP-T2A-*Antp* (Serano et al., 2004; Figure 5b). Recent work in *Parhyale* has demonstrated that even in phenotypically wild-type G0 animals, targeted alleles have a high rate of mutations within the germline, and loss-of-function mutations can be revealed by crossing these phenotypically wild-type G0 animals to each other and analyzing their G1 progeny (Kao et al., 2002). Moreover, NHEJ-mediated repair has also been used to generate transgenic animals (Kao et al., 2002). By using a double-stranded donor template without homology arms, one can generate fused transcripts at a higher rate than via a homologous recombination pathway. More recent protocols in other systems have shown that an alternate single-stranded DNA repair pathway can generate transgenic alleles at much higher efficiencies than homologous recombination (Chen, Pruett-Miller, & Davis, 2018; Quadros et al., 2010). Moreover, the identification and engineering of other CRISPR-Cas systems, including those that target RNA, or modify specific bases, has created a diverse toolkit for targeted genetic manipulations (Abudayyeh et al., 2007; Cox et al., 2005; Gaudelli et al., 2009; Komor, Kim, Packer, Zuris, & Liu, 1986). Future work in *Parhyale* will benefit from adapting additional advances in CRISPR-Cas targeting technologies to this emerging research system.

## 4 | GENOMIC AND BIOINFORMATIC RESOURCES

Gene sequence and its analysis are instrumental to many molecular genetic approaches. For example, in situ hybridization requires the generation of labeled antisense probes from specific sequences of interest, while transgenesis using CRISPR-Cas9 requires sequences for specific targets in the genome. In newer research systems such as *Parhyale*, identifying conserved genes is critical for comparative developmental genetic studies. Moreover, examining genome content and organization in *Parhyale* can provide insights into arthropod and metazoan genome evolution.

The initial identification of gene sequences in *Parhyale* relied on low-throughput approaches, such as degenerate PCR and RACE (Browne et al., 2009; Price & Patel, 2012; Prpic & Telford, 2010). The extensive efforts of early investigators generated sequences for the *Hox* genes and many other developmentally important *Parhyale* genes for performing expression and genetic analysis.

Analysis of large genomic sequences was spurred on by the construction of a BAC library consisting of 129,024 clones with an average insert size of 140 kb (Parchem et al., 2013). This library amounted to five genome equivalents of the ~3.6 Gb genome. Analysis of specific genes and gene families, including sequencing of several selected BAC clones, revealed several features of *Parhyale*, which could be compared to those in other arthropods specifically and metazoans more broadly. For example, the intergenic distance in the *Parhyale* genome is much greater than those of previously sequenced arthropods. Furthermore, analysis of coding regions in *Pax* group III and other genes revealed significantly greater intron size. These results are consistent with the very large genome size of *Parhyale*, and suggest that *Parhyale* has undergone genome expansion.

Transcriptome data has also been generated from various stages of embryogenesis and adult tissues (Blythe et al., 2012; Zeng et al., 2002). GO-term analysis of embryonic transcripts revealed no significant enrichment in genes of a particular function relative to *Daphnia* and *Drosophila*, while BLAST revealed an enrichment in transcripts containing a C2H2 zinc finger domain (Zeng et al., 2002). Blythe et al. (2012) generated a great deal of additional transcriptome data, including two additional small RNA libraries from early embryos (S1–S4) and slightly later embryos (S4-germband) to identify 55 conserved microRNA (miRNA) sequences. Two miRNAs conserved to *Eumetazoa* (miR-10 and miR-100) and 9 out of 30 miRNA families conserved to *Bilateria* were identified in *Parhyale* using this approach.

A recently published paper on a *Parhyale* genome assembly describes in deeper detail the genomic features of this crustacean (Kao et al., 2002). Genomic DNA from a single adult male was used to generate a genome assembly containing 133,015 scaffolds (N50 of 81,190 bp), 259,343 unplaced contigs, and 584,293 shorter contigs that could represent polymorphic regions. Annotation by Augustus, which uses high-confidence gene models generated using assembled transcriptomes, gene homology, and ab initio predictions, generated 28,155 gene models. Combined genomic and second-generation Illumina transcriptomic data accounted for 247 of the 248 CEGMA (Core Eukaryotic Genes Mapping Approach) orthology groups, which are a measure of assembled genome completeness (Parra, Korf, & Bradnam, 2008). Moreover, 96% of new transcriptome reads map to the genome. Taken together, this suggests that the current genome assembly captures nearly all of the protein-

coding information for *Parhyale*. This genome sequence is publically available at NCBI (https://www.ncbi.nlm.nih.gov/genome/15533).

Further transcriptomic and genomic analysis was carried out to identify noncoding RNAs, such as piwi-interacting RNAs, miRNAs, and long non-coding RNAs, as well as their associated proteins. *Parhyale* has key components of all major small RNA pathways, including four argonaute family members, two PIWI family members, and orthologs of *Dicer-1*, *Dicer-2*, *drosha*, and *loquacious* (Kao et al., 2002). However, no evidence was found for *SID-1*, a necessary component of systemic RNAi that is found in *C. elegans* and many species of mammals, fish, and insects. These findings will allow for investigations of small RNA pathway evolution through studies in a crustacean.

The *Parhyale* genome assembly revealed several additional genomic features. Analysis of transcriptomes representing *Parhyale* cultures from different labs, although all derived from the original population extracted from the Shedd Aquarium, revealed high levels of heterozygosity in many genes (Kao et al., 2002), a finding consistent with polymorphisms observed in overlapping BAC sequences (a mean of 1.5–2.5% single nucleotide polymorphisms). Moreover, analysis of BAC sequences revealed many large (~100 bp) indels between haplotypes. Consequently, greater care must be taken when using homology-dependent genetic manipulations such as CRISPR-Cas9 in this system.

Recently, the *Parhyale* community funded an updated genome sequencing effort, which included Dovetail Chicago and Hi-C methods for stitching together large contigs and bridging gaps over repetitive elements. This effort has resulted in a dramatically improved genome sequence, which will facilitate deeper investigations into the regulatory information contained in the *Parhyale* genome.

Taken together, there is now a wealth of genomic and bioinformatics data for *Parhyale*, including BACs, a high-quality genome sequence, assembled transcriptomes for various stages of development, and annotated small RNAs. Such resources will enable deeper genetic investigations in this emerging research system.

## 5 | CASE STUDIES

With thoroughly characterized development, many methods for genetic manipulation, and ample genomic resources, *Parhyale* has become a platform for a diverse portfolio of investigations. Here, we examine two topics that highlight the advantages of using *Parhyale* as a research system.

### 5.1 | *Hox* genes and body plan diversity

First discovered in *Drosophila*, the *Homeotic* or *Hox* family of genes has become appreciated as a conserved mechanism for A–P patterning of animal body plans. These transcription factors are usually found in a collinear genomic cluster that corresponds with their expression along the anterior–posterior (AP) axis (William & Krumlauf, 2008). Given their broad conservation and critical developmental function, *Hox* genes have been used to explore arthropod body plan diversity. Gene expression data for *Hox* genes is available for all of the major arthropod clades, including chelicerates, myriapods, crustaceans, and many diverse species of insects (Hughes & Kaufman, 2010; partially summarized in Figure 6b). These data suggest a correlation between *Hox* gene expression and body plan evolution. However, given the difficulties of homologizing body regions between different types of arthropods, it has been difficult to determine how exactly *Hox* expression and body plan evolution are related.

Within the arthropods, crustaceans offer an interesting opportunity for *Hox* gene analysis, as they exhibit immense diversity, particularly in regards to limb morphology, yet have body regions that can be readily homologized (VanHook & Patel, 2016), at least within some clades. Crustacean *Hox* gene expression domains correlate with the presence of particular limb types, suggesting that AP body regionalization is controlled by *Hox* expression. However, until recently, there has been no functional validation of this hypothesis. Furthermore, when looking across crustacean body plans, changes in *Hox* gene expression also correlate directly with changes to body plan organization. This suggests that changes to *Hox* gene expression are responsible for body plan evolution in crustaceans (Abzhanov & Kaufman, 2009a, 2009b, 2009c, 2000b; Averof & Patel, 2009d; Hughes & Kaufman, 2010).

With its unique status as a crustacean with advanced genetic tools and genomic resources, *Parhyale* has become an excellent system for studying crustacean *Hox* genes. Recent studies in *Parhyale* have provided additional crustacean *Hox* expression data, and the first forays into crustacean *Hox* gene function in *Parhyale* using transgenesis and CRISPR-Cas9 mutagenesis have offered strong support for previous hypotheses of *Hox*-mediated crustacean body plan evolution.

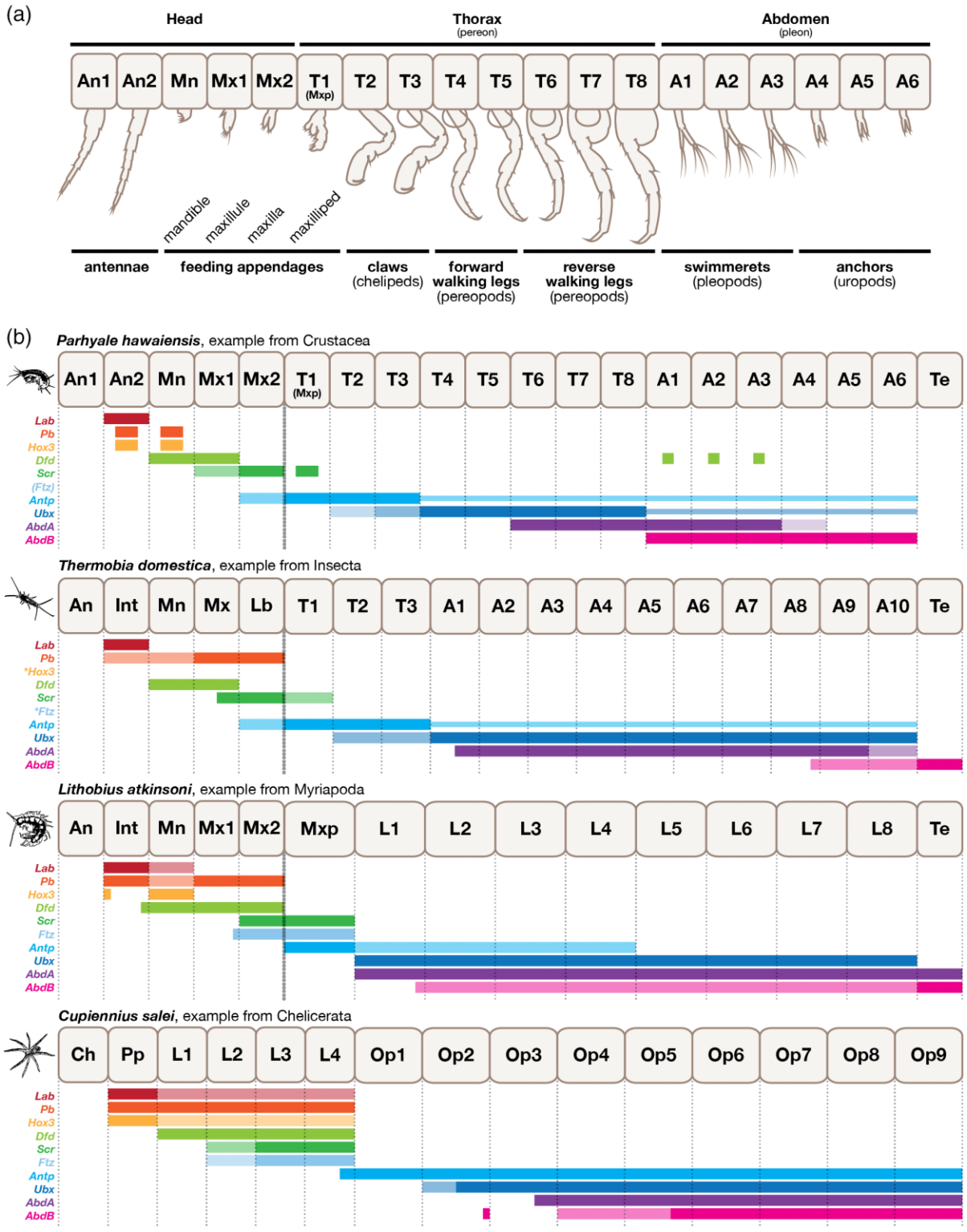


**FIGURE 6** Body plan organization and *Hox* gene expression of *Parhyale* and other representative arthropods. (a) Body plan of *Parhyale*. (b) Body plan and *Hox* gene expression of *Parhyale* and several representative arthropods. The expression of *Hox* genes correlates strongly with differing segment identities across the arthropods. *Thermobia*, *Lithobius*, and *Cupiennius* patterns were reprinted with permission from Hughes and Kaufman (2010). Copyright 2002 Elsevier Ltd

The first investigations into *Parhyale Hox* genes focused on *Parhyale Ultrabithorax* (*Ph-Ubx*). In malacostracan crustaceans, this *Hox* gene was previously correlated with the transition between feeding type appendages in the anterior thorax (maxillipeds) and the locomotory appendages found more posteriorly on the thorax. Previous work has demonstrated that a

posterior shift in the expression of *Ubx* correlates with an increase in the number of maxillipeds across a variety of crustaceans, suggesting that the removal of *Ubx* from a given thoracic segment allows for the emergence of maxilliped identity (Averof & Patel, 2009d).

In *Parhyale*, *Ubx* is expressed in segments T2–T8, which include the chelipeds, forward walking legs, and reverse jumping legs (Liubicich et al., 2012). The T1 segment in *Parhyale* has a reduced size and modified morphology, and is classified as a maxilliped. RNAi knockdown of *Ubx* induces a transformation of the T2 and T3 segments (normally chelipeds, or claws) towards a T1/Mxp-like identity, a result in agreement with previous evidence that suggested that loss or reduction of *Ubx* expression could generate maxilliped morphology. In a complementary experiment, Pavlopoulos et al. (2014) described the effects of *Ubx* misexpression using a heat-shock inducible *Ubx* construct. Ectopically induced *Ubx* expression in the antenna, Mx2, Mxp, and T2–T3 (chelipeds) produced a homeotic transformation into T4–T5 (forward walking legs). Frequently, Mx2 could be transformed into Mxp, which the authors correlated with repression of the more anteriorly expressed *Hox* gene *Ph-Sex combs reduced* (*Scr*). Moreover, *Ubx* misexpression caused overall transformation of head segments into thoracic segments. These two studies were the first demonstration that *Hox* genes in *Parhyale* were necessary and sufficient to establish regional identity in the embryo. Moreover, they provided the first functional evidence for the hypothesis that changes in *Hox* expression could be responsible for changes in crustacean body plan organization.

A comprehensive analysis of the *Parhyale Hox* genes was first provided by Serano et al. (2004) (summarized in Figure 6b). The authors initially identified the Hox genes through degenerate PCR and RACE from cDNA. Using these sequences to probe the *Parhyale* BAC library allowed for the isolation of genomic regions containing *labial*, *proboscopedia*, *Hox3*, *Deformed*, *Sex combs reduced*, *Antennapedia*, *abdominal-A*, and *Abdominal-B*, in addition to the *Ubx* gene previously described. The only ancestral arthropod *Hox* gene that appeared to be missing from *Parhyale* was *fushi tarazu.* Overlapping BACs from this original dataset determined that *lab*/*pb* and *Dfd*/*Scr*/*Antp*/*Ubx* were clustered together. Recent work using fluorescent in situ hybridization to detect nascent *Hox* transcripts has shown that the majority of the *Hox* genes are found in a single cluster, with the exception of *Hox3* (Kao et al., 2002). The 2018 *Parhyale* genome assembly places all of the *Hox* genes in a single collinear cluster that spans approximately 3.5 Mb.

Expression analysis of each of the *Hox* genes was performed at several stages of development using in situ hybridization and immunofluorescence using cross-reactive antibodies (Serano et al., 2004). In summary, the *Hox* genes are expressed with temporal and spatial collinearity: four *Hox* genes are expressed in the head: *lab*, *pb*, *Hox3*, and *Dfd*; two *Hox* genes are expressed in both the head and the thorax: *Scr* and *Antp*; *Ubx* is expressed in the thorax; *abd-A* is expressed in the thorax and abdomen, and *Abd-B* is expressed in the abdomen alone. Anterior genes are expressed earlier in development (e.g., *lab* and *Dfd* are first expressed at S8, ~16 hpf), while more posterior genes are expressed later, depending on their position along the *Hox* cluster (e.g., Antp is first expressed at S12, ~56 hpf, while Abd-B is first expressed at S17–S18, around ~86 hpf). The expression patterns identified for *Parhyale* add to the catalog of crustacean *Hox* gene expression data from crustaceans with diverse body plans, including from the branchiopods *Artemia* and *Daphnia*, the maxillopod *Sacculina*, and the malacostracans *Porcellio*, *Procambarus* and *Asellus*, thus enabling further comparisons of gene expression between species (Abzhanov & Kaufman, 2009a, 2009b, 2009c, 2000b; Averof & Akam, 1995; Blin et al., 2003; Brena et al., 2005; Copf et al., 2003; Mouchel-Vielh et al., 2002; Papillon & Telford, 2007; Shiga et al., 2002; Vick & Blum, 2010).

To interrogate how *Hox* genes specify regional identity in *Parhyale*, Martin et al. (2015) used both CRISPR-Cas9 mutagenesis and RNAi knockdown, examining the posterior six of the nine *Parhyale Hox* genes: *Dfd*, *Scr*, *Antp*, *Ubx*, *abd-A*, and *Abd-B* (summarized in Figure 7). For each CRISPR-Cas9 mutagenesis experiment, the authors independently tested two sgRNAs to control for off-target effects. In summary, knockout of each of the *Hox* genes produced various homeotic transformations that revealed their function in specifying regional identity (Figure 7). For example, knockout of *abd-A* caused a transformation of T6–T8 reverse jumping legs to T4–T5 forward walking leg identity, as well as a transformation of A1–A3 swimming legs to A4–A6 anchoring leg identity. This suggests that *abd-A* is necessary for the establishment of swimming leg and anchoring leg identity, and that these leg identities are created as a result of overlapping expression of *abd-A* with either *Ubx* or *Abd-B.* Together, the homeotic transformations identified in these experiments describe the "*Hox* code" in *Parhyale* that dictates how combinations of *Hox* gene expression generate different limb types.

By examining the expression patterns of *Hox* genes in other crustaceans, then, one can begin to make predictions about how the Hox codes in these crustaceans function to produce diverse body plans. For example, using a cross-reactive antibody (FP6.87) targeting *Ubx*/*abd-A* proteins, Martin et al. (2015) were able to discern that the abd-A expression boundary is two segments more posterior in the decapod crustacean *Procambarus fallax* and the mysid *Mysidium columbiae.* This shift in the posterior abd-A expression boundary correlates with an increased number of swimming legs. Based on the CRISPR-Cas9

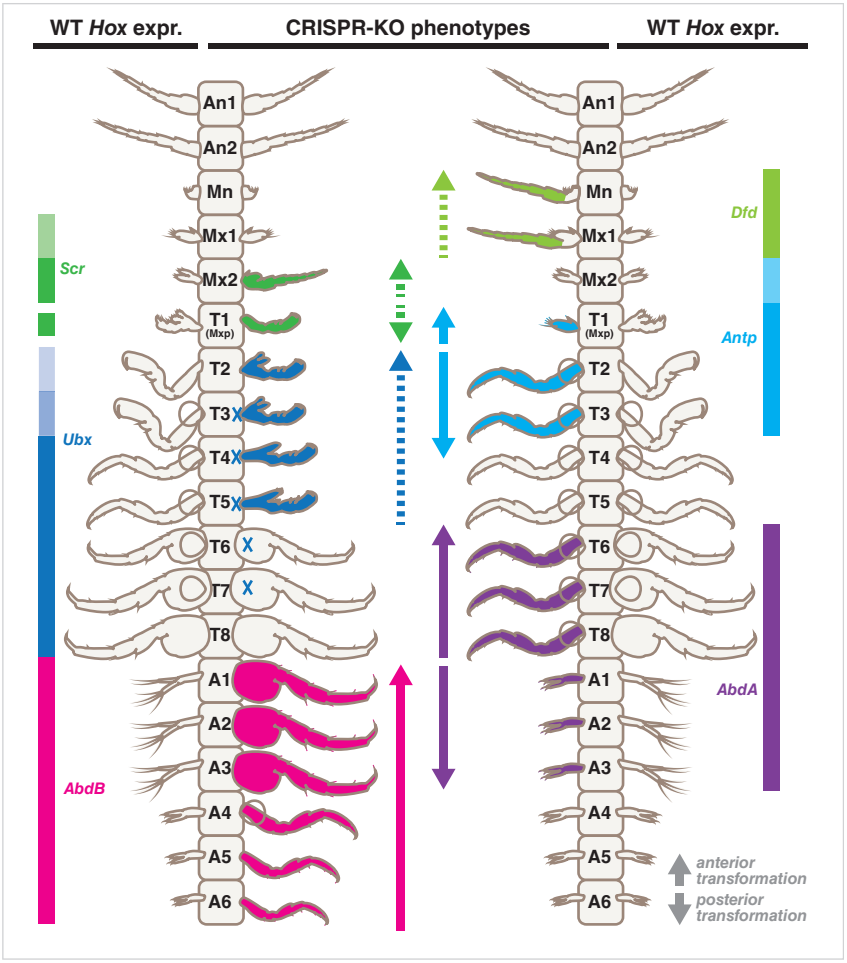


**FIGURE 7** Homeotic transformations of segment identity in *Parhyale* through *Hox* gene knockout**.** CRISPR-Cas9 knockout of *Hox* genes induces a range of different homeotic transformations, revealing the roles of these genes in specifying segment identity

phenotype of *abd-A* in *Parhyale*, one can infer that the change in pleopod number is directly induced by a change in the expression boundary of *abd-A*.

The present achievements in *Hox* gene analysis in *Parhyale* illustrate its particular advantages as a research system. In particular, the ability to perform molecular genetic manipulations (including RNAi and CRISPR-Cas9) allows for thorough analysis of gene function. As a crustacean, its complex body plan posits many avenues of future inquiry, including how Hox proteins act in combination to generate diverse limb types, and identification of genes downstream of the Hox genes that direct limb morphogenesis. Understanding *Hox* gene regulation will also help us understand further details of how evolutionary changes in limb morphology have been achieved.

## 5.2 | Regeneration

Regeneration is a widespread phenomenon found across animals. However, there does not appear to be a unifying theme to all regenerative processes, as different clades demonstrate different regenerative mechanisms and potentials. In vertebrates, such as the zebrafish, *D. rerio*, and the axolotl, *Ambystoma mexicanum*, lineage-restricted progenitors contribute to regenerated organs, whereas in planarians, a population of pluripotent stem cells is responsible for regeneration (Tanaka & Reddien, 2012). Unlike each of these species, humans lack any extensive ability to regenerate. What, then, are the molecular and genetic factors that humans have lost, and regenerative animals share?

The current research organisms available for regenerative research offer particular challenges. For example, while axolotls exhibit immense regenerative potential in multiple tissues, their large genome size and slow development make genetic research a challenge. Moreover, the regenerative strategies of planarians appear to differ substantially from those of the vertebrates that have been studied. To develop a broader picture of the ancestral regenerative state, one might wish to examine more diverse species within the evolutionary tree.

*Parhyale* is an arthropod capable of adult limb regeneration, and thus presents an excellent opportunity for examining regenerative mechanisms in a previously unexplored phylogenetic position. Early research into the mechanisms of limb regeneration in *Parhyale* has offered new insights into the ancestral urbilaterian regenerative state. Recently, Konstantinides and Averof (2009) used *Parhyale* to describe a common cellular basis for muscle regeneration between arthropods and vertebrates. In axolotls, limb regeneration appears to be lineage-restricted: dermis can make skin or cartilage, but not muscle, while muscle cells are derived from a population of *Pax3/7+* muscle stem cells called "satellite cells" (Kragl et al., 2007). Konstantinides and Averof capitalized on the early germ layer restriction of *Parhyale* embryos to label different germ layer-specific populations of cells using transposon-mediated lineage labeling. For example, by injecting a GFP-expressing *Minos* transposable element and transposase mRNA into El or Er blastomere cells, the authors labeled thoracic ectoderm cells. By amputating lineage-labeled limbs, they showed that ectodermal lineages only give rise to ectodermal cells. Meanwhile, injection into ml and mr cells labeled mesodermal lineages, including muscle. After injury, regenerated mesodermal tissue was derived from the mesodermal lineage. The authors did not identify any blastomeres that contributed to both the mesodermal and ectodermal lineages. This is also consistent with previous evidence for germ layer restriction following embryonic blastomere ablations (Price et al., 2014). Moreover, regeneration was also restricted locally to the portion of the body that was lineage-labeled; for example, ml cells could not contribute to mesodermal regeneration on the right side of the animal. These results suggest that regeneration in *Parhyale* is lineage-restricted, and that *Parhyale* does not have a general pool of lineage-restricted progenitors in the body that contribute to regeneration.

Upon deeper examination, the authors noticed a small population of DsRed-positive mesoderm-lineage-labeled cells associated with muscle fibers, which resembled satellite cells. Using a Pax3/7 antibody known to cross-react across many species (Davis, D'Alessio, & Patel, 2017), they identified these muscle-associated cells as a Pax-3/7+ population of satellite-like cells (SLCs). EdU labeling revealed that these cells also proliferate in response to limb amputation. Moreover, transplantation of fluorescently lineage-labeled muscle cells into unlabeled limbs produced fluorescently labeled muscle fibers in regenerated limbs. Together, these data suggest that *Parhyale* SLCs have similar function to satellite cells found in vertebrates. This result reveals that a homologous cell type between vertebrates and arthropods participates in limb regeneration, suggesting more broadly that the common ancestor of arthropods and vertebrates had a muscle-regeneration program that employed Pax3/7-expressing SLCs.

A recent paper by Alwes, Enjolras, and Averof (2016) has leveraged the unique optical properties of *Parhyale* to live-image limb regeneration. As *Parhyale* limbs are small and highly transparent, it is possible to observe limb regeneration in real time at the level of single cells. This optical transparency allows for live lineage tracking that is not possible in larger vertebrate regenerative systems. To visualize the regenerative process, the authors glued transgenic *Parhyale* expressing either fluorescent histones or lineage-restricted markers within a live-imaging chamber, securing the amputated limb in place. These animals largely survived the procedure, and it was then possible to image the limb during the course of regeneration, for up to 4–5 days at a time. The authors used fluorescent microscopy to examine the migration and division of individual cells, as well as the broader morphogenesis of the regenerating limb. By tracking individual cells, the authors were able to determine that all epidermal cells were equally able to participate in the regenerative process; there is no specialized cell type responsible for regeneration. Moreover, cells largely retained their original proximodistal positions along the limb as they proliferated, but changed in their positional identities upon limb differentiation and morphogenesis; for example, cells originally found in the middle of the limb, but at the edge of the amputation site, would ultimately give rise to the distal tip of the regenerated limb.

By taking advantage of the unique phylogenetic position, optical qualities, and developmental strategies of *Parhyale*, researchers have been able to develop a deeper understanding of properties common across regenerative species. This unique research system is thus poised to become a platform for novel discoveries about animal regeneration for years to come.

## 6 | CONCLUSION: FUTURE DIRECTIONS

Since its introduction in the late 1990s, the amphipod crustacean *P. hawaiensis* has provided opportunity for a wealth of comparative developmental investigations. *Parhyale* has many qualities that make it an excellent research organism, including its ease of care, easily accessible embryos, and mosaic development. With an increasingly sophisticated molecular genetic toolkit and continued proliferation in genomic and bioinformatics resources, *Parhyale* is accessible to any researcher who wishes to capitalize on its unique qualities as a research system.

*Parhyale* has already been used to investigate the evolution of early development, gene function, body plans, and regeneration. What is in store for the future of *Parhyale*? During a 2016 conference at the Howard Hughes Medical Research Institute's Janelia Farm Campus, organized by Anastasios Pavlopoulos and Michalis Averof, researchers interested in topics from

lignocellulose digestion to tissue forces to circadian rhythms presented early findings and ideas for new projects in *Parhyale*. Others proposed novel transcriptomic datasets and functional genomic approaches to study *Parhyale* development. Moreover, the *Parhyale* community has begun to amalgamate protocols, bioinformatic tools, and resources to aid additional work in this system. In the last several years, a number of papers have been posted to bioRxiv and published in other journals that explore topics as diverse as the evolutionary origins of insect wings, developmental lineage tracing, and neuroanatomy. As more researchers continue to adopt *Parhyale* into their laboratories and examine unexplored aspects of its biology, it will be exciting to see what other research explorations will be enabled by this unique emerging research system.

## ACKNOWLEDGMENTS

Aaron Pomerantz generated images of adult *Parhyale* used in Figure 1. We thank John Gerhart for inviting us to submit this review.

## CONFLICT OF INTEREST

The authors have declared no conflicts of interest for this article.

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