The crustacean model *Parhyale hawaiensis*

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Arthropods are the most abundant and diverse animals on earth. Among them, pancrustaceans are an ancient and morphologically diverse group, comprising a wide range of aquatic and semi-aquatic crustaceans as well as the insects, which emerged from crustacean ancestors to colonise most terrestrial habitats. Within insects, *Drosophila* stands out as one of the most powerful animal models, making major contributions to our understanding of development, physiology and behaviour. Given these attributes, crustaceans provide a fertile ground for exploring biological diversity through comparative studies. However, beyond insects, few crustaceans are developed sufficiently as experimental models to enable such studies. The marine amphipod *Parhyale hawaiensis* is currently the best established crustacean system, offering year-round accessibility to developmental stages, transgenic tools, genomic resources, and established genetics and imaging approaches. The *Parhyale* research community is small but diverse, investigating the evolution of development, regeneration, aspects of sensory biology, chronobiology, bioprocessing and ecotoxicology.

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Natural habitat and lifecycle

Where *Parhyale* live: *Parhyale hawaiensis* (Dana, 1853) (Peracarida, Amphipoda, Hyalidae) are marine amphipod crustaceans, belonging to the talitrid superfamily (commonly known as beach-hoppers, sand-hoppers or sand-fleas) (figure 1). First described from the Hawaiian islands (Dana 1853), *Parhyale hawaiensis* are cosmopolitan amphipods with a worldwide, circumtropical distribution (Shoemaker 1956, Barnard 1965) and might represent a species complex (Myers 1985). They occur in intertidal habitats such as bays, estuaries and mangroves (Shoemaker 1956, Poovachiranon et al. 1986), and prefer rocky beaches and macroalgal fauna (Barnard 1965). Occupying shallow intertidal waters, *Parhyale* can tolerate variations in salinity (5 to 40 ppt) and temperature (Poovachiranon et al. 1986). As effective detritus feeders with large population sizes (up to 7000 individuals per m^2) they can play an important role in ecosystems such as mangrove forests (Poovachiranon et al. 1986).

Morphology: *Parhyale* exhibit a pattern of segmental organization that is common to malacostracan crustaceans, including head, thoracic (pereon) and abdominal (pleon) units with conserved numbers of segments. The head bears sensory antennae and feeding appendages. The pereon is composed of eight segments: a first segment bearing modified feeding appendages (maxillipeds, T1), followed by segments bearing two grasping appendages (gnathopods, T2-3) and five walking legs (T4-8). Maxillipeds are morphologically and functionally associated with the head segments. Walking legs T4-5 are oriented anteriorly, while T6-8 are oriented posteriorly – hence the name 'amphipoda', which derives from the Greek αμφι/both and πους/leg. The pleon is composed of six segments, each with a pair of branched (biramous) appendages. The first three pairs are swimmerets, the following three are smaller and more stiff uropods for holding onto or jumping from the substrate. All these appendages are considered to be serially homologous (Liubicich et al. 2009, Pavlopoulos et al. 2009, Martin et al. 2016, Pavlopoulos and Wolff 2020). Divakaran (1976) provides a detailed description of the morphology, organ systems and biology of *Parhyale hawaiensis*.

Lifecycle: Like most amphipod crustaceans, *Parhyale* shows sexual dimorphism. Mature males are usually larger than females of the same age and bear T3 gnathopods in which the last two leg segments (propodus, dactylus) are significantly enlarged. Males use this enlarged claw-like structure to clasp and hold on to a female in a mating position known as amplexus (figure 1). *Parhyale* adults pair a few days before females are ready to lay eggs. When the female molts, the male transfers sperm into the female's brood pouch and releases her, as she starts to release her eggs in the brood pouch (Divakaran 1976, Borowsky and Borowsky 1987). As in most peracarid crustaceans, the females carry the fertilized eggs in a ventral brood pouch (marsupium), which is formed by specialized side branches (oostegites) of thoracic appendages T3-6 (Wolff and Gerberding 2015). Depending on the size and the age of the female, the marsupium can contain up to ~30 eggs. The eggs of each brood are developmentally synchronized (Browne et al. 2005). The embryonic development is direct, with no larval stages. After about 10 days at 26°C, juveniles hatch as miniature versions of the adult (Browne et al. 2005) and stay for a few more days inside the marsupium. *Parhyale* grow through successive molts, in which they shed their cuticle and replace it by a new one, during their entire lifetime. Their body length increases from ~1 mm in hatchlings to >10 mm in adults. Secondary sexual characters, such as oostegites and male claspers differentiate during reproductive maturation, which takes at least 6-7 weeks at 26°C.

Fig. 1. *Parhyale hawaiensis* couple, with a large male clasping a smaller female. (Photo credit: Vincent Mongorgé)

From mangrove to lab

Historical info: While *Parhyale* are found in tropical waters around the world, the lab cultures of *Parhyale* are derived from animals collected by William Browne from the seawater filtration system of the Shedd Aquarium (Chicago, IL, USA) almost twenty-five years ago, in 1997. Thus, the geographical origin of the cultured animals remains a mystery, as the *Parhyale* presumably entered the system as hitchhikers from other material purposely introduced into the seawater tanks.

The selection of an amphipod crustacean was not a complete accident, however, as the extensive work of Wolfgang Dohle (reviewed in Dohle and Scholtz 1988) had documented a remarkable pattern of orderly cell divisions that generates the germband of amphipod crustaceans, and at that time there was great interest in understanding the process of segmentation in arthropods that, unlike *Drosophila*, add segments sequentially in a cellularised rather than a syncytial context. Thus, *Parhyale* was an ideal organism for comparative evolutionary developmental studies of early pattern formation (phylogenetic context presented in figure 2). Once introduced into the lab, *Parhyale* were readily established in shallow seawater tanks and easily maintained with relatively little effort (Gerberding et al. 2002). There were some issues with rotifers in the female brood pouches that led to high mortality of embryos, but these rotifers were eventually removed from the cultures.

Lab culture: *Parhyale* are very hardy and do well in a variety of culture systems (figure 3). They are generally kept in shallow trays of artificial seawater with either an airstone or impeller pump to keep water circulating. They will readily accept a variety of food including fish flakes, kelp powder, carrots, and pellets formulated for feeding shrimp. A substrate composed of crushed coral or aragonite rock will help to buffer the pH and provide hiding spaces for juvenile animals. Water changes at two-week intervals help to maintain water quality, although animals will easily survive even under conditions of poor water quality provided that the water remains well oxygenated. Water salinity is kept at 24-32 ppt. Tanks with an active biological filtration system and strong circulation can support many hundreds of adult animals per liter. Under ideal conditions of 26-28°C with frequent feeding, *Parhyale* can go through an entire generation in about two months. Individual adults can survive and remain reproductive for up to 2-3 years. *Parhyale* cultures are so robust that the species is being considered for aquaculture (Vargas-Abúndez et al. 2021).

Inbred line: Shortly after the *Parhyale* cultures were established, an inbred isofemale line, Chicago-F, was created through two rounds of sibling inbreeding, and then maintained as an isolated population since. This line was used for the creation of a BAC library (Parchem et al. 2010, at the time the line was called Iso2) and for genome sequencing (Kao et al., 2016). Further attempts at inbreeding resulted in unhealthy animals which failed to survive (M. Vargas-Vila and N. Patel, unpublished observations). The Chicago-F line carries fewer polymorphisms than other laboratory populations, but still carries a high level of polymorphism in some parts of the genome (Kao et al., 2016).

Major interests and research questions

Embryonic development and germ layer formation: Embryogenesis takes ~11 days at 26°C. Embryos undergo direct development, so hatch as miniature versions of the adults. Eggs can be removed from the female brood pouch without harming the female, and the eggs are easily maintained in petri dishes with sterile seawater.

Embryogenesis has been divided into a series of stages based on morphological criteria (Browne at el. 2005). Initial cell divisions are holoblastic, and at 8 hours of development the embryo is composed of 8 cells: 4 macromeres and 4 micromeres. Initial lineage studies show that these cells subsequently give rise to defined germ layers of the embryo (Gerberding et al. 2002, Price et al. 2010). The four macromeres are called Ep, El, Er, and Mav, and the four micromeres are g, mr, ml, and en. Ep, Er, and El give rise to the ectoderm (posterior, right, and left, respectively). Mav gives rise to the visceral mesoderm and anterior somatic mesoderm. The somatic mesoderm on the left and right sides of the body are derived from ml and mr respectively, and g gives rise to the germline. en was initially characterized as producing the endoderm, although subsequent studies suggest that it gives rise to extra-embryonic cells and the endoderm is derived from Mav (C. Chaw and N. Patel, unpublished). This lineage pattern is also observed in another amphipod, *Orchestia cavimana* (Wolff and Scholtz 2002).

Ablation experiments of Ep, Er, El, ml, mr, and Mav reveal that compensation is possible when an individual macromere or micromere is ablated, but that compensation is restricted to within a given germ layer (Price et al. 2010). Thus, ablation of an ectodermal blastomere will be compensated, but only by another blastomere that would normally also give rise to ectoderm, and the same is true for the mesoderm. This compensation occurs if the ablation is done to a

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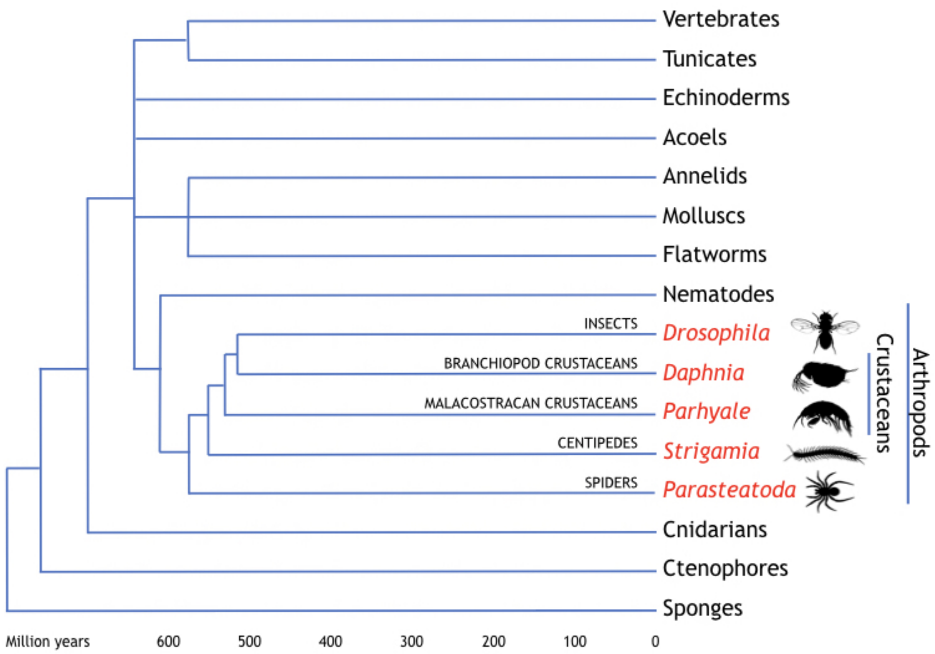

Fig. 2. *Parhyale* and other arthropod models, shown in the context of animal phylogeny, focusing on experimental and/or genomics models representing five major arthropod clades: insects, branchiopod and malacostracan crustaceans, centipedes and spiders. Crustaceans are paraphyletic with respect to the insects (Schwentner et al. 2017). The divergence times are tentative and in some cases controversial; unresolved nodes are shown as polytomies (see Telford et al. 2015, Kapli and Telford 2020).

blastomere or its progeny prior to gastrulation. Once gastrulation has occurred, compensation either does not occur, or is very incomplete (Price et al. 2010; Hannibal et al. 2012a). Studies of the g blastomere suggest that this cell contains germ plasm (Ozhan-Kizil et al. 2009, Gupta and Extavour 2013) and other lineages cannot compensate for g cell ablation during embryogenesis (Extavour 2005).

The ectoderm of the thorax and abdomen form by condensation of the ectodermal cells into an orderly array of rows and columns, which then follow a remarkably well-organized pattern of subsequent divisions, so that each subsequent parasegment of the embryo is derived from a single row of precursor cells, called a parasegment precursor row (PSPR) (Gerberding et al. 2002; Browne at al. 2005; Sun and Patel 2019). This pattern of parasegment formation is also seen in other crustaceans (reviewed in Dohle and Scholtz 1988), although amphipods are unique amongst malacostracan crustaceans in not possessing ectoteloblast stem cells that produce the PSPRs. The somatic mesoderm of the thorax and abdomen of Parhayle are produced by eight mesotelobast stem cells (four per side) derived from ml and mr. These mesoteloblasts undergo a series of highly organized asymmetric divisions, which form rows of mesoblasts, with each row of mesoblasts then giving rise to a segmental unit of muscles (Gerberding et al. 2002; Hannibal et al. 2012a, Hannibal et al. 2012b).

Body and limb patterning: Like most other crustaceans, *Parhyale* possess a pair of appendages on each segment of the body. Orthologs of the homeotic (*Hox*) genes have been identified in *Parhyale* and found to be expressed along the anterior-posterior axis of the embryo as in most other animals (Liubicich et al. 2009, Serano at el. 2016). They are present as a single complex spanning about 3.6 Mb (D. Sun and N. Patel, unpublished observations). The function in *Parhyale* of one of these genes, *Ultrabithorax* (*Ubx*), has been probed by ubiquitous misexpression using a transgenic approach (Pavlopoulos et al. 2009) and knockdown via RNAi (Liubicich et al. 2009). Both experiments confirmed that Ubx plays a role in establishing segmental identity, most obviously seen by transformations of appendage identity. Furthermore, these studies supported the previous hypothesis, based on comparison of Ubx protein expression across several crustacean lineages, that Ubx plays a role in distinguishing maxillipeds from more typical locomotory appendages in crustaceans (Averof and Patel 1997).

More recently a systematic analysis of *Hox* gene function was carried out using CRISPR knockouts (Martin et al. 2016), which led to a detailed picture of how the identity of segments of the thorax and abdomen is specified. The results are largely consistent with the role of *Hox* genes in specifying segmental identities, as seen in insects, but many of the details suggest that there are important evolutionary differences in the expression patterns and in the interaction between *Hox* genes in *Parhyale* and *Drosophila* (Martin et al. 2016, Serano et al. 2016). Most notably, the general pattern of posterior prevalence is not obeyed for *Ubx*, *abd-A*, and *Abd-B* (further supported through an analysis of double mutant combinations; E. Jarvis and N. Patel, unpublished data). These results provide a basis for analysing the potential role of *Hox* genes in evolutionary changes in body patterning across crustacean lineages.

Arthropods also display a clear regionalization along the proximal-distal axis of their limbs, which appears to be mediated by an evolutionarily conserved set of genes in all arthropods. CRISPR-based knockout of *Parhyale* orthologs of these leg patterning genes suggests a specific evolutionary relationship of individual leg segments between *Parhyale* and insects, which supports the hypothesis that proximal leg segments present in the last common ancestor of insects and crustaceans became incorporated into the body wall during insect evolution. This change may explain the evolutionary origin of insect wings from a proximal exite (Bruce and Patel 2020). Studies on the orthologues of *Drosophila* wing patterning genes in *Parhyale* and in other crustaceans provide additional insights on the complex evolutionary origins of insect wings (Clark-Hachtel and Tomoyasu 2020, Shiga et al. 2017).

Cell lineage analyses of outgrowing thoracic limbs show that the early limb primordium becomes subdivided into anterior-posterior and dorsal-ventral compartments whose boundaries intersect at the distal tip of the growing limb. Limb formation is associated with a spatial modulation of cell proliferation. Limb elongation is driven by preferential orientation of cell divisions along the proximal-distal growth axis (Wolff et al. 2018).

Regeneration: *Parhyale* have the ability to regenerate their limbs, following injuries sustained by attacks from predators, cannibalism, or disease. They are able to regenerate any appendage (antennae, mouthparts, thoracic limbs, pleopods and uropods) and retain that ability throughout their lifetime.

Since their body is enclosed within a chitinous exoskeleton, arthropods depend on molting to fully restore the structure and function of their regenerating limbs. Live imaging using fluorescent nuclear markers has shown that the following events take place once a *Parhyale* leg has been amputated: 1) within minutes from the cut, haemocytes adhere to the wound surface and form a plug that stops the bleeding, 2) the wound surface becomes melanised within the next hours, a typical wounding reaction of arthropods that is mediated by the haemocytes at the wound, 3) within 1-2 days the wound epithelium stretches and closes over the wound surface, under the melanised scab, 4) extensive cell proliferation takes place in the region of the wound, 5) the epithelium at the wound site detaches from the cuticle and limb morphogenesis takes place below the scab, 6) the animal molts, the newly regenerated limb is released and becomes functional (Alwes et al. 2016). Studies in other crustaceans suggest there is a complex interplay between the timing of regeneration and molting (Charmantier-Daures and Vernet 2004), but this crosstalk has not yet been systematically investigated in *Parhyale*.

Many malacostracan crustaceans, including crabs and lobsters, can self-amputate limbs that are severely injured, a process known as autotomy. The injured limbs are broken off at specific sites near the base of the limb, under the control of a nervous reflex (Fredericq 1882). We have never directly observed autotomy in *Parhyale*. Limbs can be amputated at any site along their proximo-distal axis, where they will heal and regenerate the missing parts.

Research in *Parhyale* has so far focused on the cellular basis of leg regeneration. Studies in mosaic animals, where individual blastomere lineages were marked by transposons carrying a fluorescent marker, established that *Parhyale* limb regeneration relies on distinct pools of progenitor cells for ectoderm and mesoderm, and that these progenitors reside locally, near the site of regeneration (Konstantinides and Averof 2014). There are no totipotent progenitors.

Cell transplantation and live imaging experiments have revealed the cellular sources for regeneration of muscle and epidermis: mesodermal cells resembling the satellite cells of vertebrates appear to act as progenitors for muscle (Konstantinides and Averof 2014), while any epidermal cell near the site of amputation appears capable of regenerating the epidermis (Alwes et al. 2016).

A unique asset for studying leg regeneration in *Parhyale*, so far unparalleled in other experimental models, is the ability to image regeneration continuously, at single-cell resolution (Alwes et al. 2016, Sugawara et al. 2021). This approach allows cell lineages to be tracked over the entire course of regeneration, from the time of amputation to the subsequent molt (~1 week), and provides a basis for studying the cell behaviours that underpin morphogenesis in regenerating legs. Detailed transcriptional profiling of leg regeneration has been performed in *Parhyale* (C. Sinigaglia, M. Paris and M. Averof, unpublished), but to our knowledge no relevant studies of gene function have been undertaken so far. The impermeable cuticle that surrounds the limbs presents a significant challenge for *in situ* hybridization and immunochemical stainings.

Neurobiology, sensory systems and behaviour: The central nervous system of *Parhyale* is composed of a brain and ventral nerve cord. The adult brain contains about 13,300 nuclei in total (Wittfoth et al. 2019), a small number which renders this brain tractable for neuroanatomical studies. The ventral nerve cord comprises a fused subesophageal ganglion, seven segmental ganglia innervating the pereon, three segmental ganglia innervating pleon segments 1-3, and one fused ganglion innervating pleon segments 4-6 (Divakaran 1982).

The brain of *Parhyale* is tripartite and formed during development through condensation of three segmental cephalic neuromeres. They represent three morphological regions with segmental identity: the protocerebrum, associated with the compound eyes, the deutocerebrum, associated with the first antenna, and the tritocerebrum, associated with the second antenna (Divakaran 1982). The general architecture of all major neuropils is in agreement with the suggested ground-pattern of malacostracan crustaceans (see Kenning et al. 2013), however, some of these neuropils are difficult to demarcate due to their close association and uniform appearance. For example, in the visual system there are uncertainties about the presence/absence of the lobula and the inner chiasma connecting the medulla (Ramos et al. 2019, Wittfoth et al. 2019). Studies of photoreceptor projections to the optic lobe neuropils have revealed some unexpected differences from a conserved pattern of photoreceptor projections found in other crustaceans and insects, but these differences remain poorly understood (Ramos et al. 2019).

Although there is significant anatomical conservation across amphipod brains, there is also a degree of variability in the construction and size of different neuropils (Hanström, 1932), which seems to correlate with ecology and life history (Ramm and Scholtz 2017). The brain of *Parhyale* does not display any major modifications or bias towards one sensory modality, therefore it likely represents a common type of amphipod brain.

The visual system of *Parhyale* consists of a pair of eyes, with up to ~50 ommatidia per adult eye, and the underlying visual circuits (Ramos et al. 2019). Hatchlings start off with a small number of ommatidia, which then grow in size and in number, likely increasing visual sensitivity and the resolution several fold as the animals grow to adulthood (see Keskinen et al. 2002).

Each ommatidium contains five photoreceptor cells, four with large rhabdomeres (R1-4) and one with a small rhabdomere (R5). These two types of photoreceptors express distinct opsins and project their axons to distinct regions of the first optic neuropil (Ramos et al. 2019). Photoreceptors R1-R4 have long, well-aligned rhabdomeres that should make them intrinsically sensitive to the direction of light polarisation. The rhabdomeres of R1+R3 and R2+R4 are arranged in perpendicular orientations, which would make them most sensitive to different directions of polarised light. Overall, this design gives *Parhyale* very low visual resolution (20° interommatidial angle and 50 resolved pixels per adult eye, compared with 5° and 700 pixels in *Drosophila*, Land 1997), but the potential to detect colour and polarised light (Ramos et al. 2019).

These results suggest that *Parhyale* do not use vision to perform tasks that require good spatial resolution, such as finding food or interacting with mates (e.g. see Holmes 1903), but are likely to use vision to orient themselves within their habitat, e.g. responding to gradients of light intensity and polarisation. Behavioural experiments in other talitrid amphipods support this notion (Ercolini and Scapini 1976, Forward et al. 2009, Cohen et al. 2010, Ugolini et al. 2012, Cohen and Putts 2013, Ugolini 2014, Ciofini et al. 2020). Simple behavioural experiments in *Parhyale* suggest that they have a phototactic response (Ramos et al. 2019).

Photoreceptors lacking overt pigmentation and a light-focusing apparatus have been found in the brain of other talitrid amphipods (Frelon-Raimond et al. 2002). These photoreceptors could perform non-visual functions, such as entraining circadian rhythms. We speculate that in *Parhyale* these cells could be marked by the expression of the *3xP3* marker (see below).

Parhyale are likely to use chemosensory cues rather than vision to locate their food and mating partners. In laboratory conditions it is noticeable that males are more active in searching for females and probing whether they are receptive to mating (C. Wolff, personal observation). This observation is in agreement with studies on intertidal amphipod populations (Alegretti et al. 2016) and suggests that waterborne attractants or contact pheromones might play an important role in reproductive behavior. There are studies on sexual dimorphisms of the peripheral sensory system, suggesting that the first antennae of male amphipods can be equipped with male-specific sensilla (Lowry 1986, Hallberg et al. 1997).

Circadian and tidal rhythms: *Parhyale* has recently attracted attention as an experimental model for studying biological clocks. Initial studies focused on probing circadian rhythms in locomotor activity, establishing a head transcriptome at 3-hour intervals across the 24-hour day/night cycle, and identifying components of the circadian clock, including Bmal1/Cycle, Cry2 and Per (Hunt 2016, Hunt et al. 2019). The genome of *Parhyale* appears to lack orthologues of Cry1 and Timeless (Hunt et al. 2019). Preliminary observations on *Parhyale*'s locomotor behaviour revealed 24-hour and 12-hour rhythms of activity, with highest activity in the dark hours of the circadian cycle (Hunt 2016). As an organism living in intertidal habitats, *Parhyale* can also serve as a model for studying circatidal rhythms (E. Kwiatkowski, J. Rosenthal and P. Emery, unpublished observations).

Lignocellulose digestion: Insects such as termites and beetles have evolved complex digestive systems with the ability to digest wood and to use it as their main source of energy. To achieve this, they rely on populations of specialized symbiotic microbes, resident in their digestive tract, which provide essential enzymatic functions to digest lignocellulose (Watanabe and Tukoda 2010). Within malacostracan crustaceans, some isopods and amphipods, including *Parhyale*, have evolved the capability to digest lignocellulose without relying on such microbes (Zimmer et al. 2002, Cragg et al. 2015). The *Parhyale* genome encodes GH7 family glycosyl hydrolase enzymes that are necessary for hydrolytically digesting lignocellulose (Kao et al. 2016). This offers an opportunity to understand the activity of glycosyl hydrolases in a digestive system, independently of gut microbes.

Lignocellulose (plant) biomass is the most abundant raw material on earth and exploiting this energy source on a larger scale would offer an exceptional chance to produce biofuels (Himmel et al. 2007). The genetic tools available in *Parhyale* could make significant contributions to this research.

Ecotoxicology: Amphipods are used as indicator or test species for environmental toxicology in aquatic habitats (e.g. Kunz et al. 2010, Poynton et al. 2018). *Parhyale* are emerging as an attractive ecotoxicity test organism for coastal marine ecosystems, due to their worldwide distribution and abundance in tropical coastal and estuarine habitats, and their robust year-round reproduction in their natural habitats and in the laboratory (Alegretti et al. 2016, Artal et al. 2018). Recent studies have tested the effects of ammonia, metals, the

Fig. 3. A dense laboratory culture of *Parhyale* feeding on shrimp meal wafers.

pesticide diflubenzuron and silver nanoparticles in this system (Artal et al. 2018, Artal et al. 2020, Diehl et al. 2021).

Experimental tools, approaches and resources

Experimental embryology: Cell ablation experiments can be carried out following a variety of methods in *Parhyale*. Injection of FITC-dextran into blastomeres is not harmful as long as embryos remain in subdued light, but exposure to intense blue light will kill cells containing the FITC-dextran. This method has been used to kill individual cells at the 4- and 8-cell stage, or entire lineages at gastrulation or even at germband stages (Price et al. 2010, Hannibal et al. 2012a). Cell ablation can also be accomplished by injection of RNase and DNase (Chaw and Patel 2012). Manual ablations are also possible by poking a hole into the target cell and removing the cell content (Alwes et al. 2011). Finally, individual cells, or small groups of cells, can also be efficiently killed by laser ablation; this technique has been used to examine the role of the *Parhyale* midline in establishing dorsal-ventral patterning of the germband (Vargas-Vila et al. 2010) and to probe the role on nerves in limb regeneration (C. Sinigaglia and M. Averof, unpublished).

Individual blastomeres can be separated and maintained in short term culture to probe their developmental potential in isolation from their neighbours (Extavour 2005).

Robust protocols have been established for antibody staining (Rehm et al. 2009a) and *in situ* hybridization (Rehm et al. 2009b) of *Parhyale* embryos. A number of antibodies are available, including some made against *Parhyale* protein sequences, as well as cross-reacting antibodies raised against conserved epitopes. Recently, *in situ* HCR has been used to probe multiple transcripts per embryo (Bruce and Patel 2020). Using HCR it is possible to simultaneously image five different transcripts at once, as well as to achieve robust results at hatchling stages when the cuticle has formed (H. Bruce and N. Patel, unpublished).

Loss-of function approaches: Three approaches have been described for knockdown/knockout of gene expression in *Parhyale*: RNAi, morpholino, and CRISPR-Cas9. For RNAi, injection of Stealth siRNAs into 1-2 cell embryos was used to knockdown expression of *Distal-less* and *Ultrabithorax* (Liubicich et al. 2009) and *single-minded* (Vargas-Vila et al. 2010). Stealth siRNAs were found to be more efficient than dsRNA, possibly due to their increased stability, allowing them to persist to later stages of development. As is typical with RNAi experiments, this approach produces a phenotypic series, which can help to understand gene function. It also appears that RNAi acts cell-autonomously, as injection into a single blastomere at the 2-cell stage could create half-mutant embryos (D. Liubicich and N. Patel, unpublished observations). Integration of a *Minos*-based heat-inducible transgene generating a hairpin RNA of *abd-A* was also successfully used for knockdown and provides an approach for temporal control of RNAi in *Parhyale* (Martin et al. 2016).

The injection of morpholinos has been used successfully to inhibit the translation of *vasa*, which resulted in the death of germ cells (Ozhan-Kizil et al. 2009).

More recently, CRISPR-based approaches have been used to knock out a number of genes (Martin et al. 2016, Kao et al. 2016, Clark-Hachtel and Tomoyasu 2020, Bruce and Patel 2020). As in other systems, injections of Cas9 protein along with guide RNA is more effective than injections of Cas9 mRNA with guide RNA (Martin et al. 2016), and chemically modified sgRNAs (Synthego) have proven particularly efficient (Bruce and Patel 2020; E. Jarvis and N. Patel, unpublished observations). CRISPR knockouts allow for rapid phenotypic analysis as the gene edits occur early and (when using the most effective gRNAs) with such efficiency that fully mutant G0 animals can be readily generated. The technique can also be applied to analyze lineage specific gene function through injection at the 8-cell stage (E. Jarvis and N. Patel, unpublished).

Transgenesis: Transgenesis is routinely carried out in *Parhyale* using the *Minos* vector (Pavlopoulos and Averof 2005, Kontarakis and Pavlopoulos 2014), a versatile transposon that has been used with success in a wide variety of organisms (including insects, chordates and fungi, see Pavlopoulos et al. 2007, Evangelinos et al. 2015). The vector typically carries a marker and a cargo that can be many kb in length. *Minos* plasmid constructs are co-injected with transposase mRNA in early embryos; the transposon jumps randomly in the *Parhyale* genome, giving rise to mosaic individuals (G0), carrying *Minos* insertions in different parts of the body. Once the transposase activity dies out, the insertions are fixed and thereafter inherited as stable genetic elements (Pavlopoulos and Averof 2005).

As with RNAi and CRISPR, the slow holoblastic cleavage and stereotypic early cell lineage of *Parhyale* offer some unique advantages, particularly the ability to test transgene constructs in G0s and the opportunity to generate mosaics where only half of the embryo or a specific germ layer is genetically marked/modified (see Pavlopoulos et al. 2009, Konstantinides and Averof 2014).

Typically, about a third of embryos injected at the 1-cell stage give rise to mosaic G0s. Only a fraction of *Minos* insertions are present in the germline and will be carried through to subsequent generations (G1, G2, etc.). Transgenic individuals are identified through the marker carried in the transposon. For a long time transgenesis was based on *3xP3*-driven fluorescent markers (Pavlopoulos and Averof 2005), previously shown to be active in the eyes of several insects (Berghammer et al. 1999, Horn and Wimmer 2000, Uhlirova et al. 2002). In *Parhyale*, *3xP3* markers are not expressed in the eyes, but in a pair of spots at the posterior of the head (Pavlopoulos and Averof 2005). In recent years, a *Parhyale* opsin promoter (*PhOpsin1*) has been adopted for marker expression, which drives robust expression in the eyes and is much easier to score (Ramos et al. 2019).

Transgenic *Parhyale* lines often carry tens of genomic insertions of *Minos* (Pavlopoulos and Averof 2005) and are maintained as mixed 'stocks', in which individuals carry different sets of insertions. This speeds up the process of estab-

Fig. 4. Transgenic *Parhyale* juvenile expressing EGFP in muscles under the *PhMS* promoter. Confocal image showing EGFP (green) and cuticle autofluorescence (white).

lishing transgenic lines, but results in heterogeneous stocks in which the frequency of individual insertions can drift over time.

Alternative transgenic approaches are available in *Parhyale*, in which the transgenes are targeted to specific loci rather than randomly integrated in the genome: 1) using the *phiC31* integrase to target transgenes to specific landing sites inserted in the genome (Kontarakis et al. 2011), and 2) using CRISPR knock-ins (Serano et al. 2016, Kao et al. 2016). Both could be useful for establishing transgenic lines carrying single, mapped insertions, but neither is yet routinely established. The *phiC31* approach requires establishing *Parhyale* lines with well characterised landing sites (ongoing work in the Pavlopoulos lab).

Genetic markers and drivers: Transgenesis in *Parhyale* has so far been used to fluorescently mark cells for live imaging and to build genetic tools for manipulating gene function. Both applications require the identification of functional *cis*-regulatory elements (CREs) to drive the expression of marker and effector proteins. Two types of CREs have been tested in *Parhyale* using reporter constructs: artificial elements consisting of multimerized binding sites for specific transcription factors, known to work in other species, and candidate CREs from *Parhyale* genes.

Among artificial elements, the Pax6-responsive *3xP3* element combined with the *Drosophila hsp70* core promoter (Berghammer et al. 1999, Horn and Wimmer 2000) drives weak expression in a few cells at the posterior of the brain (Pavlopoulos and Averof 2005). The weak expression and lack of expression in the eyes may be due to the use of a heterologous core promoter from *Drosophila*. A second element, *DC5*, consisting of Pax and Sox2 binding sites (Kamachi et al. 2001, Blanco et al. 2005; combined with the *PhHS* promoter region), drives strong expression in the central nervous system. In the strongest lines, *DC5* also marks the motor neurons of the limbs (Konstantinides and Averof 2014, Alwes et al. 2016). Reporters with multimerised Pax3/7 binding sites (Relaix et al. 2003) were also tested but not found to be functional in *Parhyale* (M. Grillo and M. Averof, unpublished).

So far, four robust endogenous CREs have been identified in *Parhyale*, two from *hsp70* family genes and two from opsins. The first to be identified, *PhMS* (MS for muscle-specific), is a fragment cloned from a *Parhyale hsp70c* (*hsp70*-related) gene, which drives robust expression in muscles (Pavlopoulos and Averof 2005, sequence accession FR749990) (figure 4). It includes upstream regions that contain an array of putative bHLH binding sites, the promoter and a 5' UTR with an intron (see fig. S1 in Kontarakis et al. 2011). *PhMS* has been used to visualize muscle fibres and satellite-like muscle precursors in the context of leg regeneration (Konstantinides and Averof 2014). The second, *PhHS* (HS for heat shock), is a heat inducible CRE cloned from a *Parhyale hsp70* gene (Pavlopoulos et al. 2009, sequence accession FM991730). The fragment includes upstream sequences with putative HSF binding sites, a promoter and a 5' UTR with an intron (fig. S1 in Kontarakis et al. 2011). *PhHS* is routinely used to drive ubiquitous and inducible gene expression (e.g. Alwes et al. 2016, Wolff et al. 2018). Finally, the *PhOpsin1* and *PhOpsin2* CREs, derived from the *Parhyale opsin 1* and *opsin 2* genes, drive expression in each of the two types of photoreceptor cells in the eyes of *Parhyale* (Ramos et al. 2019). *PhOpsin1* is now used to drive robust expression of transgenic markers, replacing the less reliable *3xP3* markers.

In addition to these robust CREs, numerous other gene fragments have been tested for CRE activity unsuccessfully (Averof, Pavlopoulos and Patel labs, unpublished). These include gene fragments upstream of ubiquitously expressed housekeeping genes, developmental genes and viral promoters, in some cases guided by chromatin profiling via ATAC-seq. The failure to identify active CREs in these cases could be due to poor genome annotation (e.g. mis-identification of promoter regions), the wide dispersal of CREs in *Parhyale*'s large genome, the relatively small size of the tested fragments (usually no larger than 5 kb), or a bad choice of core promoters.

Several core promoters that have been tested in *Parhyale* (including the *Drosophila hsp70*, *Parhyale PhHS* and the artificial *SCP* core promoters), but none appeared to work reliably with heterologous CREs (A. Pavlopoulos, A. Kiupakis, M. Grillo and M. Averof, unpublished).

An alternative approach for generating markers is to generate enhancer or exon traps, in which expression of a transgene is driven by endogenous *cis*-regulatory/transcriptional activity at the site of insertion. *Minos* constructs bearing a core promoter sequence upstream of a fluorescent marker have given putative enhancer traps (F. Alwes, C. Cevrim and M. Averof, unpublished) and elements carrying a splice donor have been shown to mediate exon trapping in *Parhyale* (Kontarakis et al. 2011). Enhancer or exon traps can be repurposed into diverse genetic tools using the *phiC31* integrase, if they contain an *attP* site (Kontarakis et al. 2011), or by using CRISPR-mediated transgene replacement (Gilles et al. 2015).

The large average size of introns in *Parhyale* (Kao et al. 2016) may particularly facilitate exon trapping in this species. Exon trap screens have produced up to 2 trap lines per 100 injected embryos in *Parhyale* (Kontarakis et al. 2011, and F. Alwes unpublished). However it has been challenging to establish and maintain large numbers of exon trap lines.

A further alternative is to use CRISPR-mediated gene editing to knock-in marker or effector proteins in loci with the required pattern of gene expression (see Serano et al. 2016, Kao et al. 2016). The efficiency of CRISPR-mediated knock-ins can be very low (depending on target locus and gRNA) and careful consideration should be given to the knock-in strategy, to facilitate the selection of insertions and to avoid deleterious effects at the target locus.

Genetic tools based on transgenesis: Transgenesis provides opportunities to establish a wide range of genetic tools and approaches in *Parhyale*. First and foremost, the ability to express genes for gain-of-function studies. Since most genetic interventions are likely to have deleterious effects on survival, establishing conditional systems for gene expression has been a top priority. The *PhHS* regulatory element (described above) currently provides a robust tool for inducible gene expression in *Parhyale*, typically induced by a heat-shock of 30-60 minutes at 37°C (Pavlopoulos et al. 2009). So far, besides the expression of fluorescent markers, *PhHS* has been used to mis-express different isoforms of the *Hox* protein Ubx, leading to homeotic transformations (Pavlopoulos et al. 2009). One flaw is that the *PhHS* element gives some variable/leaky expression in the absence of a heat shock, particularly in muscles.

Developing a binary system for mis-expressing genes, such as the GAL4/UAS system that has revolutionised studies in *Drosophila* (Brand and Perrimon 1993), has been the focus of several unsuccessful tests in *Parhyale* (A. Pavlopoulos, A. Kiupakis, V. Douris and M. Averof, unpublished; J. Serano and N. Patel, unpublished). Possible reasons for the lack of success so far may include the lack of an effective core promoter, or the variable/insufficient activity of GAL4 observed in some species (e.g. Asakawa et al. 2008, Schinko et al. 2010).

Some attempts have also been made to develop recombinase-mediated clonal approaches in *Parhyale* (N. Konstantinides, M. Grillo and M. Averof, unpublished). So far, only the *phiC31* integrase has been shown to work efficiently (Kontarakis et al. 2011) and a version of the *Raeppli* clonal marker which is based on this integrase (Kanca et al. 2013) has given promising early results (M. Grillo, P. Ramos and M. Averof, unpublished).

Various genetic approaches for inducible cell killing have been developed in different model organisms. One of the most versatile approaches combines transgene-mediated expression of the nitroreductase/NTR protein (providing spatial or cell-type specificity) with the application of the prodrug metronidazole to kill the NTR-expressing cells (Curado et al. 2008). Preliminary tests, using the *PhMS* and *DC5* regulatory elements to drive nitroreductase expression, indicate that this system provides an effective conditional approach for ablating specific cell types in *Parhyale* (I. Koltsaki and M. Averof, unpublished; C. Winchell and N. Patel, unpublished).

Live imaging and cell lineage analysis: The availability of fluorescent markers, the small size and the relative transparency of embryos, juveniles and adults, render *Parhyale* attractive for live imaging. The first live imaging and lineage analysis experiments in *Parhyale* were carried out by injection of fluorescently labelled high molecular weight dextrans or mRNA encoding fluorescent proteins between the 1- to 16-cell stage (Gerberding et al. 2002, Rehm et al. 2009c, Price and Patel 2007, Price et al. 2010, Chaw and Patel 2012, Hannibal et al. 2012a, Hannibal et al. 2012b). With these injection approaches, however, fluorescence signal fades with time due to a combination of dilution by cell division and degradation of the fluorescent reporter. With injections in individual blastomeres at the 8-cell stage, signal remains clearly visible until hatching for the g cell (germline) lineage, owing to the very few cell divisions in this lineage, while in the ectoderm or mesoderm reliable cell tracking is only possible until mid germband stages (approx. stage 19-21). Cell lineages up to the gastrulation stage have also been imaged and tracked in unlabelled embryos using Nomarski optics (Alwes et al. 2011).

More recently, live imaging experiments have used stable transgenic lines expressing histone-H2B-tagged fluorescent proteins to track cells during embryonic development and regeneration (Alwes et al. 2016, Wolff et al. 2018, Sugawara et al. 2021). The fusion with histone allows the nuclei of individual cells to be resolved and be tracked through mitoses. Imaging at 5-20 minute time intervals is sufficient to detect mitoses and to track dividing cells during embryonic development and regeneration. Additionally, to visualise the cell cycle, a reporter of the S, G2 and M phases of the cell cycle has been developed by fusing EGFP with the N-terminus of *Parhyale* Geminin (Alwes et al. 2016).

Live imaging is still constrained by the limited number of markers available in *Parhyale*. In the absence of a characterised constitutive promoter, ubiquitous markers rely on the *PhHS* regulatory element, which requires repeated heat-shocks to sustain marker expression.

Continuous live imaging has been performed over long periods in *Parhyale* using fluorescence widefield microscopy (spanning embryonic development, Price and Patel 2007, Hannibal et al. 2012a), light-sheet microscopy (4-5 days on mid-late embryos, Wolff et al. 2018), and confocal microscopy (2-11 days on regenerating adult limbs, Alwes et al. 2016, Sugawara et al. 2021, C. Cevrim and M. Averof, unpublished). Over extended periods of imaging photodamage becomes a concern, especially when imaging on a confocal microscope. Steps to minimise light exposure include imaging strong fluorescent signals with low laser power, minimizing the number of imaged z planes and lengthening time lapse intervals, which constrain the spatial and temporal resolution of imaging. Under these conditions, *Parhyale* can be imaged over multiple days and give rise to fully developed embryos or regenerated limbs. Light-sheet microscopy reduces fluorophore bleaching and phototoxic effects to a large extent (Stelzer 2014). *Parhyale* embryos can be easily mounted in agarose (Price and Patel 2007; Wolff et al. 2018).

Cell tracking has been performed on time lapse recordings in early embryogenesis (Alwes et al. 2011; Chaw and Patel, 2012), segment formation (Price and Patel, 2007; Hannibal et al. 2012a), limb development (Wolff et al. 2018) and adult limb regeneration (Alwes et al. 2016, Sugawara et al. 2021), in some cases yielding complete cell lineages that span several days of development and regeneration. Leading software for cell tracking – MaMuT/Mastodon (Wolff et al. 2018) and ELEPHANT (incorporating deep learning, Sugawara et al. 2021) – have been developed and tested on *Parhyale* light-sheet and confocal data.

Examples of live image recordings can be found at the following links, for ectoderm and mesoderm segmentation (Hannibal et al. 2012a), limb development (Wolff et al. 2018) and limb regeneration (Sugawara et al. 2021).

Genetic crosses and forward genetics: Single-pair genetic crosses are performed routinely in *Parhyale* to establish transgenic lines and to combine transgenes. The females do not store sperm from previous matings, so there is no need to collect virgins. The lack of sperm transfer has been confirmed using fluorescent transgenic markers (G. Tsoumpekos and M. Averof, unpublished).

While establishing transgenic lines in *Parhyale*, we have in some cases identified heritable defects in embryonic patterning, eye morphology and eye colour, likely to be caused by spontaneous mutations arising in our laboratory populations (P. Ramos and M. Averof, unpublished). These mutations were recessive and/or deleterious for survival, so we were not able to maintain them over long periods of time. The lack of easy solutions for maintaining stocks (such as cryopreservation), the slow reproductive rate (small brood size) and the long generation time of *Parhyale* make systematic forward genetics in this species a distant dream.

Genome sequence: The size of *Parhyale*'s genome is 3.6 Gb, similar to the size of the human genome and ~20 times larger than the genome of *Drosophila*.

The building of *Parhyale* genomic resources started in the early 2000s, at a time when sequencing gigabase-size genomes required huge investments. A BAC library of *Parhyale* genomic DNA with ~5x coverage of the genome was generated, screened and partly sequenced (Parchem et al. 2010), allowing to identify important developmental genes (Hannibal et al. 2012b, Serano et al. 2016) and later to validate the full genome assembly (Kao et al. 2016).

The sequences of the *Parhyale* genome were obtained from a single male of the Chicago-F inbred line, using high throughput short read technology (Kao et al. 2016). The initial published assembly, named Phaw3.0, was one of the largest sequenced genomes at the time. It revealed high levels of heterozygosity, consistent with the high frequency of polymorphism found in lab populations. A large fraction of the genome was found to consist of repetitive sequences (57% of the assembled scaffolds), which at least partly explains the large size of the genome. High polymorphism and repeat content present serious challenges for genome assembly and caused this initial assembly to be somewhat fragmented (scaffold N50 of ~70 kb, scaffold L50 of ~14000, https://www.ncbi.nlm.nih.gov/assembly/GCA_001587735.1/).

The genome assembly was subsequently updated by Dovetail Genomics using Chicago and Hi-C technologies (Burton et al. 2013, Putnam et al. 2016) and curated by D. Kao (sponsored by the Pavlopoulos, Patel, Averof, Extavour and Aboobaker teams). The current assembly, named Phaw_5.0, has much larger scaffolds (scaffold N50 of 20 Mb, scaffold L50 of 42), although contig length has only moderately increased (from N50 of 4 kb for Phaw3.0 to N50 of ~10 kb for Phaw_5.0). This genome assembly remains unpublished, but it is publicly available (https://www.ncbi.nlm.nih.gov/assembly/GCA_001587735.2/).

Parhyale hawaiensis have 23 pairs of chromosomes (Kao et al. 2016). Although the latest genome assembly of *Parhyale* is of good quality, full contiguous chromosomal sequences are not available. With the advent of high throughput sequencing using long reads (tens or hundreds of kb long), chromosome-scale assemblies are quickly becoming the new standard, even for crustacean genomes (e.g. Baldwin-Brown al. 2018). It is likely that long reads will be necessary to cross through islands of repetitive sequences and to resolve haplotypes in the *Parhyale* genome, resulting in much longer contigs.

The closest relative of *Parhyale* with an assembled genome is *Hyalella azteca*, whose genome assembly is significantly smaller (estimated genome size ~1 Gb, genome assembly 550 Mb) and more fragmented (N50 of ~200 kb for v2.0, Poynton et al. 2018). It is estimated that *Parhyale* and *Hyalella* have diverged for 40-90 million years (Copilas-Ciocianu et al. 2020), which means that non-functional sequences will be too dissimilar in these species to be properly aligned.

Gene identification and annotation: Before genome sequencing, several transcriptomic datasets were produced in *Parhyale*, including EST data generated by Sanger sequencing and *de novo* transcriptome assemblies generated by using early NGS sequencing technologies (454 pyrosequencing and SOLiD) on mRNA and small RNAs from maternal and embryonic tissues (Zeng et al. 2011, Blythe et al. 2012). DNA microarrays (Nestorov et al. 2013) and proteomic data (Trapp et al. 2016) were also produced to serve specific projects. Later, additional *de novo* transcriptomes were generated by Illumina sequencing to investigate embryonic development, limb regeneration, ecotoxicology and circadian rhythms in *Parhyale* (unpublished data from the Patel and Averof labs, Artal at al. 2018, Hunt et al. 2019).

When the *Parhyale* genome was sequenced, genes were annotated based on *ab initio* gene prediction (ORFs, splicing signals, etc.) and transcriptome data from diverse embryonic stages and adult regenerating limbs (Kao et al. 2016). In the Phaw3.0 assembly, many genes were split in unconnected contigs and the corresponding gene predictions were incomplete. In the Phaw_5.0 assembly, gene models were significantly improved and most manually curated genes in this dataset were found to be of good quality. However this assembly still contains errors, such as incorrect ordering or orientation of contigs within a scaffold (a known problem of Hi-C-scaffolded genomes) and tandem repetitions due to unresolved polymorphic repeats (M. Paris, unpublished observations). These problems have not been quantified genome-wide.

EST data and transcriptomes helped to reveal that a large fraction of mRNAs in *Parhyale* are subject to *trans*-splicing (Douris et al. 2010), whereby a shared leader sequence is spliced to the 5' end of diverse mRNAs (including mRNAs encoding the transcription factors Ultrabithorax, Twist and Mef2). The leader sequence comes from snRNAs that resemble spliceosomal snRNAs. Overall, we estimate that 10-20% of *Parhyale* mRNAs are *trans*-spliced (Douris et al. 2010, and M. Averof, unpublished observations).

Additional improvements in these gene models have been made by mapping a large number of new reads, obtained since the original gene models were assembled (M. Paris, unpublished data). Sequencing of long cDNAs utilizing Oxford Nanopore technology is underway to provide additional improvements to gene models and information on transcript isoforms (D. Sun, A. Pomerantz, N. Patel, unpublished). Direct long-read sequencing of RNA would also allow the detection of modified bases in the RNA (Garalde et al. 2018).

Functional annotation of the *Parhyale* gene models is still limited. Gene orthologues of major classes of signalling molecules, transcription factors, non-coding RNAs and effectors of innate immunity were identified in the initial genome assembly (Kao et al. 2016). SID-1, implicated in systemic RNAi (Winston et al. 2002, Maruekawong et al. 2018), was not found, which is consistent with the cell autonomous effects of RNAi described above.

Putative orthologies between *Parhyale*, *Drosophila* and human genes have been inferred on a genome-wide scale by reciprocal BLAST analysis (M. Paris, unpublished data) and by tree-based orthology reconstruction between *Parhyale* and 66 other metazoans (J. Huerta-Cepas and colleagues, unpublished data).

Expression and chromatin profiling: Genome-wide expression profiling by RNAseq has been applied in a number of projects to identify transcriptional dynamics and responses to treatments in *Parhyale*, including the timecourses of embryonic development (D. Sun, H. Bruce and N. Patel, unpublished) and limb development and regeneration (C. Sinigaglia, M. Paris and M. Averof, unpublished), the study of circadian rhythms (Hunt et al. 2019), the maternal to zygotic transition (S. Rawas, W. Wei, M. Jara Espejo and A. Aboobaker, unpublished), innate immune responses to bacterial infection (L. Li and A. Aboobaker, unpublished), and responses to environmental toxicants (Artal et al. 2020). Single-cell or single-nucleus RNAseq has been applied to embryos (L. Blondel and C. Extavour, unpublished) and adult limbs (A. Almazan, M. Paris and M. Averof, unpublished). The large average intron size in *Parhyale* (median of >7 kb in *Parhyale* versus 1 kb in *Drosophila* and 18 kb in humans) favours capturing both spliced and unspliced/intronic reads, which can help to probe transcriptional dynamics at the single-cell level (La Manno et al. 2018) and to work on dissociated nuclei instead of whole cells.

Besides single-cell sequencing, cell- or tissue-specific transcriptional profiling can be carried out with the thiouracil/TU tagging approach, whereby transcripts expressed in specific cells over a chosen period of time can be tagged, isolated and sequenced (Miller et al. 2009, Gay et al. 2014). The approach is based on transgene-mediated expression of uracil-phosphoribosyltransferase/UPRT (providing spatial or cell type specificity) and the application of the nucleobase analogue 4-thiouracil (providing temporal control), which is converted by UPRT into a ribonucleotide that can be incorporated in RNA. Tests of the TU-tagging approach in *Parhyale*, using mosaic embryos that express UPRT in the mesoderm or the ectoderm, yielded 40- to 80-fold enrichments of mesodermal or ectodermal transcripts, respectively (N. Konstantinides and M. Averof, unpublished).

The study of chromatin states and epigenetic modifications is only just beginning in *Parhyale*. Bisulfite sequencing has revealed widespread CpG dinucleotide methylation in the *Parhyale* genome (Kao et al. 2016). Bulk and single-cell ATAC-seq data have been generated, revealing genome-wide patterns of chromatin accessibility in embryonic and adult tissues, providing a platform for the identification of novel *cis*-regulatory elements and many additional assays in the future (E. Skafida and M. Paris, unpublished; D Sun and N. Patel, unpublished).

Research community

The *Parhyale* research community is small, consisting of no more than 20-30 researchers at any time. Informal meetings, gathering most of the community, have been organised in Tübingen (2005), Lisbon (2012) and Janelia Research Campus (2017). The community welcomes new researchers, sharing genomic resources, transgenic lines and technical expertise.

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