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Integrative physiology, functional genomics and the phenotype gap: a guide for comparative physiologists

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Summary

Classical, curiosity-led comparative physiology finds itself at a crossroads. Major funding for classical physiology is becoming harder to find, as grant agencies focus on more molecular approaches or on science with more immediate strategic value to their respective countries. In turn, this shift in funding places Zoology and Animal Science departments under enormous stress: student numbers are buoyant, but how can research funding be maintained at high levels?

Our research group has argued for the redefinition of integrative physiology as the investigation of gene function in an organotypic context in the intact animal. Implicit in this definition is the use of transgenics and reverse genetics to manipulate gene function in a cell-specific manner; this in turn implies the use of a genetically tractable 'model organism'. The significance of this definition is that it aligns integrative physiology with functional genomics. Again, functional genomics draws heavily on reverse genetics to elucidate the function of novel genes. The phenotype gap (the mismatch between what a genetic model organism's genome encodes and the reasons that it has historically been studied) emphasises the need to attract and empower functional biologists: can *all* 13 500 genes in *Drosophila* really be explained in terms of developmental biology? So, by embracing the integrative physiology manifesto, comparative physiologists can not only accelerate their own research, but their functional skills can make them indispensable in the post-genomic endeavour.

Glossary available online at
http://jeb.biologists.org/cgi/content/full/210/9/1632/DC1

Key words: *Drosophila melanogaster*, *Caenorhabditis elegans*, *Danio rerio*, mouse, Malpighian tubule, bioinformatics.

Introduction

This paper is aimed at students and academics who are contemplating whether to attempt real, integrative physiology – particularly in the fruit fly, *Drosophila melanogaster*. It sets out the reasons why this may be desirable and outlines the basic steps needed to obtain the resources required and to start experimenting.

Integrative physiology and functional genomics

Physiology is the study of how a living organism works. Classical physiologists are becoming an endangered species, just as the need for their skills is growing once again. This shortfall is for several reasons, not least the stringency of regulations for animal work but also because non-molecular work is now discouraged by many grant agencies. Nonetheless, cellular and molecular approaches to physiology have proved potent and cost-effective paradigms. Ultimately, however, these reductionist or analytical methodologies can prove restricting: how can we draw on the strengths of these methodologies, while addressing function in the whole organism – the new, 'integrative physiology'?

Our group has argued that, rather than abandoning molecular biology in a return to classical techniques, it is possible to integrate a gene-based approach into studies of the whole organism (Dow and Davies, 2003). Integrative physiology is seen – as in the present motto of the American Physiological Society – as the move '*from gene to tissue to organism*'. How can this be achieved? We have argued that transgenic organisms, in which expression of a particular gene of interest is manipulated upward or downward in a cell-specific manner, provide a potent link between gene and organism (Dow and Davies, 2003). In essence, physiologists are exhorted to adopt the skills of the geneticist, by trying to understand the function of specific genes by disrupting them or modulating them – what is known as 'reverse genetics'. This philosophy is not merely

academic: it was adopted by the UK's Biotechnology and Biological Sciences Research Council in their recent 'Genomics in Animal Function (GAIN)' Initiative.

The attraction of this working definition of integrative physiologist is that it aligns comparative physiology with one of the most important research fields today; that of functional genomics – the understanding of the function of all the genes encoded in a genome. Perhaps one-third of the genes in any genome (so perhaps 5000–10 000) are sufficiently novel that their function cannot be predicted *in silico*; for such genes, a reverse genetic work-up is considered one of the most powerful approaches.

For functional genomics, however, there is a major log jam in the reverse genetic pipeline; there is no point in mutating a particular gene unless the experimenter is able to recognize and study any resulting phenotype. Functional genomics thus requires the broadest range of functional biologists to align themselves with their endeavour. The problem is that model organisms have come to prominence, not for their physiological tractability but for highly focused studies – typically of development. The 'phenotype gap' is therefore the mismatch between the functions encoded by an organism's genome and what it has historically been used to study (Brown and Peters, 1996; Bullard, 2001; Dow, 2003; Wang et al., 2004). The scale of this mismatch for most model organisms emphasizes the opportunities available to comparative physiologists flexible enough to take on a new organism.

It is actually possible to quantify the phenotype gap. By the time the *Drosophila* genome was sequenced in 2000, I estimate that at least 300 000 researcher-years had been devoted to *Drosophila* – the large majority spent on studies of development. *Drosophila* genes are given (more or less witty) names as they are first encountered, and so it is possible to distinguish known, studied genes from those that are merely computer annotated. Of the 13 500 or so genes identified in the first release of the genomic sequence, only 20% were named (Wang et al., 2004). We can thus assume that developmental biology, as a screen for new genes, has now neared saturation. For the other 80% of genes, we need to seek new phenotypes.

The need for model organisms

Once the case for transgenics as a physiological tool is accepted, then the choice of organism is immediately circumscribed. Transgenics is only feasible in the small set of organisms (like mice, fruit flies, worms or zebra fish) known as genetic models (Table 1). Typically, these organisms have fully sequenced genomes and a wealth of freely available mutant stocks, or the resources to create more as required. However, this appears to run contrary to the Krogh principle, beloved of comparative physiologists; that for any physiological question, there is a species in which it can best be studied (Krogh, 1929). When there are perhaps 30 million species of animal (mostly insect) in the world, how can it be sensible to restrict oneself to less than a few tens of species?

The answer is twofold. Firstly, integrative physiology is not doctrinaire; it remains possible to continue working in a target species, while dipping occasionally into the remarkable resources associated with the model organism. For example, an insect physiologist working on an agricultural pest organism could look up the sequence of a particular neuropeptide receptor from the *Drosophila* genome project and use it to design primers that would be likely to pull the gene out of the target organism. Alternatively, if a gene was identified by an advanced molecular technique like suppression subtractive hybridization in the target species, mutants could be sought in the phylogenetically closest model species, and studied there, so as to understand the gene of interest rather better. The second answer is that, just because a model organism has a sequenced genome and a wealth of genetic resources, it is not intrinsically less interesting than a non-model; indeed, if one considers that the Krogh principle applies both to an organism that exemplifies a trait '*and the ease with which it can be studied experimentally*', then model organisms can acquire Krogh status for a surprising range of studies.

Real physiology

So far, these arguments could be seen as philosophical. However, taking *Drosophila* as an example, it is possible to identify several examples of real physiology interacting closely with genetics in order to provide powerful, fundamental insights.

(1) Our understanding of the circadian clock is based on pioneering (and painstaking) screens of insects that failed to eclose from their pupae at the normal time of day, implying that they had lost track of time (Konopka and Benzer, 1971). Mutants discovered in *Drosophila*, like *period* and *timeless*, have influenced the whole field.

(2) Similarly, mutants identified in simple learning paradigms have implicated the cyclic AMP signalling pathway; dunce is a cAMP phosphodiesterase, while rutabaga is an adenylate cyclase (Dudai et al., 1976; Dudai and Zvi, 1985). The work in *Drosophila* thus closely aligns with Kandel's Nobel prize-winning studies on habituation of the gill withdrawal reflex in the non-model sea hare, *Aplysia californica* (Kandel and Schwartz, 1982).

(3) The suspicion that the 24-transmembrane pass voltage-gated ion channels were actually derived from two gene duplication events acting on a 6-transmembrane pass ancestor was dramatically confirmed when a line of flies with legs that continued to shake under ether anaesthesia (Catsch, 1944) were shown to be mutants in a potassium channel gene, named *Shaker* (Kamb et al., 1987). At first sight, one might think it only appropriate that a primitive channel had been identified in a 'primitive' organism, but this would show a grave misunderstanding of evolution. *Drosophila* has continued to evolve in the 400 million years since it diverged from the common ancestor of humans, so it is *different*, rather than *primitive*. Indeed, the Shaker channel of *Drosophila* triggered the discovery of a ubiquitous family of quarter-sized channels that were found even in humans (Salkoff et al., 1992).

Of course, these physiological examples are all drawn from neuroscience. Our group's work, however, has shown that renal function can also be studied to great advantage in *Drosophila* (Dow and Davies, 2001; Dow and Davies, 2003; Dow and Davies, 2006). The sequenced genome allowed the rapid identification of genes encoding diuretic neuropeptides (Cabrero et al., 2002; Coast et al., 2001; Kean et al., 2002; Terhzaz et al., 1999) and their receptors (Johnson et al., 2005; Radford et al., 2002), often before it proved possible in non-model organisms, and indeed these studies paved the way for similar work in other insects (Radford et al., 2004).

Using the tools

The key genetic tool for *Drosophila* physiology is the GAL4 enhancer trap. This has been described in detail many times (Brand and Perrimon, 1993; Sentry et al., 1994) but, in essence, it provides the ability to express genetic constructs of choice in specific cells in an otherwise normal organism – exactly the technological requirements for integrative physiology (Dow and Davies, 2003). Targeted ectopic expression is possible in a wide range of genetic models, but perhaps nowhere as simply as in *Drosophila*. The GAL4/UAS system is binary; that is, a fly is generated by crossing together a 'driver' line (one in which the yeast transcription factor GAL4 is expressed in a desired pattern) and a UAS line (one in which the genetic payload is placed downstream of five copies of the UAS binding site recognized by GAL4). In such flies, the genetic payload is switched on strongly in those cells in which GAL4 is being expressed (Fig. 1).

Where do such useful lines come from? GAL4 drivers are either derived from enhancer trap screens or are made by inserting a gene's control regions next to the *GAL4* gene in a plasmid, which is then incorporated into the germ line of *Drosophila*. Our lab participated in the Kaiser screens (Kaiser, 1993) of the Brand constructs (Brand and Perrimon, 1993), and from 750 lines obtained 50 with patterned expression in the alimentary canal, of which about 10 provided informative and useful expression patterns in the Malpighian tubules (Sözen et al., 1997). This panel of driver lines has been of great use to us, but have also been distributed around the world to other tubule groups. Similarly, most *Drosophila* labs around the world now keep a panel of GAL4 drivers of utility to their research areas.

However, there is still greater sophistication available. What if it is desirable to express a gene of interest not just in a particular spatial pattern but at a particular time? For example, it might be important to express a deleterious construct only in adult *Drosophila* just before study, to prevent pleiotropic effects (or even lethality) in the embryo or larva. This can be achieved with a temperature-sensitive GAL4-binding protein, GAL80. In flies transgenic for a GAL4 driver, a UAS-driven transgene and GAL80, GAL4 is expressed in a spatially restricted pattern, but bound by GAL80, thus preventing it from binding to UAS and activating the transgene. However, when the temperature is raised to 30°C, GAL80 dissociates, allowing GAL4 to bind to UAS and so activate the transgene (McGuire et al., 2004; Suster et al., 2004).

Another strategy is to provide the genetic equivalent of a 'latching' switch for the GAL4/UAS system. If a fly is generated containing a GAL4 driver and a UAS-driven payload, but in the additional presence of a GAL4 transgene downstream of a UAS promoter, then wherever GAL4 is transiently expressed, it will activate the GAL4 transgene, so providing high levels of GAL4 expression indefinitely in that cell (Hassan et al., 2000).

Sometimes, it can be hard to study the effects of a gene because available mutants are lethal. The traditional *Drosophila* genetic technique is to generate X-ray-induced mosaics, in which the mutation is only homozygous in a small population of cells (Becker, 1975). Clever experimental design can allow these cells to be visibly marked; these mutant patches can then be studied in the context of an otherwise normal animal. Yeast technology adds a modern twist to these classical experiments; if the lethal mutation is crossed onto a chromosome that has a yeast flippase recombination target (FRT) site near its centromere, and then yeast flippase (FLP) is driven transgenically, there is a finite chance that the chromosomes will recombine (Golic and Lindquist, 1989; Xu and Harrison, 1994). This has the effect of producing clones of cells carrying two mutant (or two wild-type) chromosomes. Of course, if UAS–FLP is driven with GAL4, it is possible to target specific populations of cells, rather than throughout the organism.

RNA interference (RNAi) by double-stranded RNA, originally employed in *Caenorhabditis elegans* (Fire et al., 1998), has proved a potent means of obtaining hypomorphic

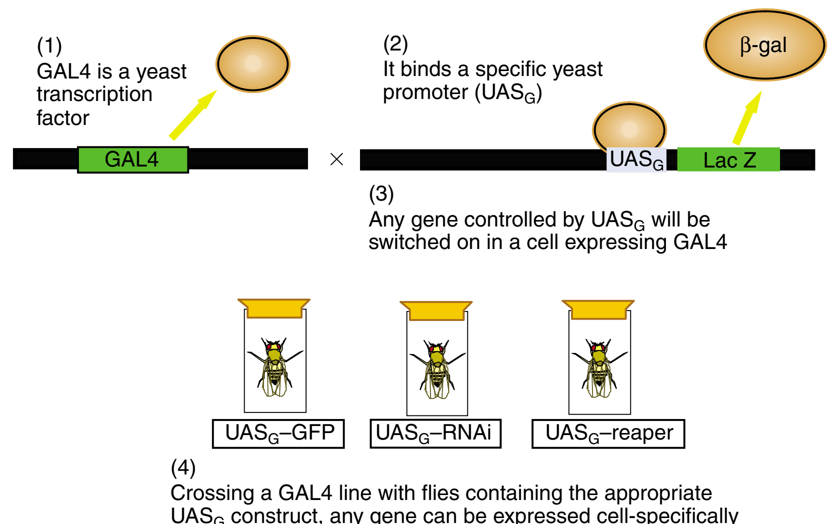

Fig. 1. The GAL4/UAS system. Modified from Dow (Dow, 2007).

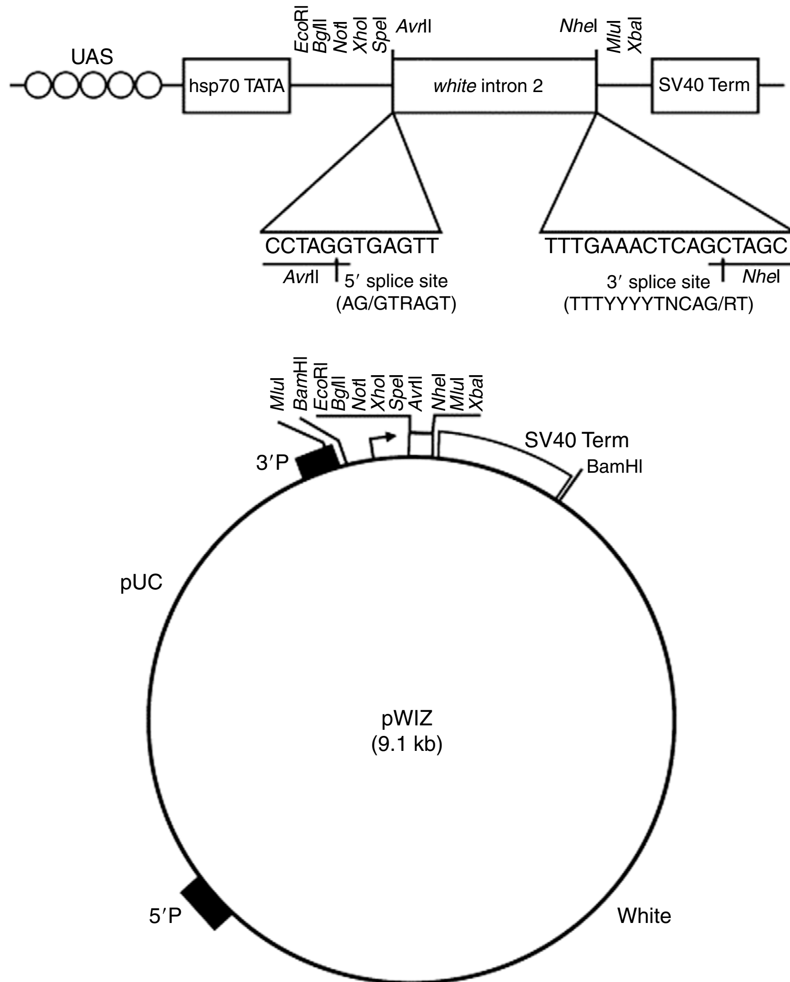

Fig. 2. Schematic of the pWiz vector. Reproduced with permission from Lee and Carthew (Lee and Carthew, 2003).

alleles of genes, without resorting to screening for new mutants. Although many means of expressing double-stranded RNA (dsRNA), the precursor of short interfering RNA (siRNA), have been developed, the favoured means at present is to use Carthew's pWiz vector (Lee and Carthew, 2003). This has a complete pUC plasmid with antibiotic resistance marker (so that it can be grown up in *Escherichia coli*), P-element ends (so that it can transform into *Drosophila* at high efficiency), a *white* minigene (to allow transgenic flies to be identified by their red eyes) and rare-cutter sites (downstream of UAS) that allow DNA fragments to be dropped into the vector in opposite orientations (Fig. 2). The cloning sites are on either side of an efficiently spliced *white* intron, believed to help in the generation of dsRNA. This makes the generation of flies transgenic for UAS–RNAi constructs a matter of a week's cloning, followed by a three-month period of intermittent fly husbandry.

The weakness of many model organisms is the difficulty or impossibility of targeted homologous recombination. Rather than just adding a transgene, this is the ability to replace a particular genomic sequence with another sequence; for example, to replace the open reading frame of a gene with green fluorescent protein (GFP), so inactivating the gene while putting the reporter under precisely the combination of promoters and enhancers that control the wild-type gene. In *Drosophila*, this is now possible, although not trivial. Golic's lab realized that the limitation was in producing a linear targeting construct capable of recombination with the *Drosophila* germ-line (Rong and Golic, 2000; Rong et al., 2002). The procedure they designed involves inserting the targeting construct stably into the germline by conventional transformation, then excising it at FRT sites with a transgenic FLP recombinase enzyme. The targeting construct is then able to synapse with its target, allowing recombination to take place with reasonable efficiency (Rong and Golic, 2000; Rong et al., 2002).

The jewel in the crown of *Drosophila* genetics, however, is its long history of community-generated resources. The largest stock centre, in Bloomington (http://flystocks.bio.indiana.edu/), holds over 20 000 mutant fly lines, available for a nominal charge by e-mail: requests are processed within a couple of weeks. Not only does this imply that there is a good chance that an informative allele for a particular gene may already exist but there is also the possibility of handling the direct descendents of flies identified by the *Drosophila* pioneers, Morgan, Bridges and Sturtevant. For example, we were recently able to document the recapitulation of the human renal disease xanthinuria type I in mutants at the *Drosophila* rosy locus, using the direct descendents of the *rosy*^{2} mutants first identified around 1916 (Wang et al., 2004). The utility of these mutants is increased by the ease with which they can be identified; the freely accessible Flybase website (http://www.flybase.net/) is exemplary in its ease of use.

Of course, similar results could be achieved in other organisms; it is the speed and cost that makes the *Drosophila* version of the technology so attractive.

Drosophila and other models

I suggest that there are certain minimum requirements on a model organism for it to be useful for integrative physiology. It must be possible to introduce transgenes, to produce mutations of specific genes and to intervene in a tissue-specific manner. It must also be possible to perform physiology on the organism and it is highly desirable that there should be a sequenced genome, as this makes many aspects of the work far easier.

In this context, it becomes clear that the trade-off between biomedical relevance and genetic power does not automatically lead to the mouse as uniformly best choice (Table 1).

Put simply, a transgenic mouse line takes several years, and perhaps in excess of $100 000 to realize, and then $10 000/year

Table 1. *Comparison of important properties of some model organisms*

Organism	Genome?	Generation time?	Cost/year?	Transgenics?	Targeted mutagenesis?	Tissue specific?	Available mutants?	Physiology?
Human	Yes	16–40 years	Very high	No!	No!	No	Yes	Very limited
Mouse	Yes	3 months	Very high	Yes	Yes	Yes	Many	Yes
Zebra fish	End 2008	3–4 months	Moderate	No	No	Yes	Yes	Some
Drosophila	Yes	1 week	Very low	Yes	Yes	Yes	Many	Some
Caenorhabditis	Yes	4 days	Very low	Yes	Yes	Yes	Many	Very little
Saccharomyces	Yes	90 min	Very low	Yes	Yes	Yes	Many	Not as we know it
Escherichia	Yes	20 min	Very low	Yes	Yes	Yes	Many	Not as we know it

to maintain thereafter; whereas a transgenic fruit-fly line can be made for $500 in three months and then costs $30/year to maintain. Clearly, an investigator can gain time and flexibility, while saving considerable amounts of money, if their questions can be addressed in a simpler model.

Neither should it be assumed that biomedical research is the only kind worth doing! Integrative physiology has perhaps as many as 30 million species with which to work; and the demands of both basic, curiosity-led research and perceived ‘usefulness’ do not have to be antagonistic. For example, many millions of lives are lost annually to parasitic diseases (notably, but not exclusively, malaria) that are carried by Diptera – phylogenetically close relatives of *Drosophila* (Butler, 2003).

More generally, is there thus an adequate phylogenetic spread of model species such that integrative biology can be evoked for a good proportion of problems in comparative physiology? So far, the match is not perfect, according to the NCBI’s genome page (http://www.ncbi.nlm.nih.gov/Genomes/). Mammals are well-represented for genome projects, and mouse is the mainstay model organism. The position is similar for insects, with *Drosophila melanogaster* as the constituency champion (and sequencing underway or complete for a total of 50 species). For birds, the chicken genome is now released, but the transgenic resources are not yet as potent as for the first models. For this reason, much is made of a particularly easy-to-transform lymphoid cell line with high rates of recombination, DT40 (Buerstedde et al., 1990) – although this of course is hardly ‘integrative’ as we have defined it. Other species, such as the zebra finch, *Taeniopygia guttata*, are being sequenced. For fish, fugu (*Takifugu rubripes*), the related pufferfish *Tetraodon nigroviridis* and zebra fish (*Danio rerio*), and *Oryzias latipes* (Japanese medaka) sequences are nearly complete; but targeted mutagenesis has been announced several times, rather than been deployed as a routine tool, in fish. Elasmobranchs are represented by *Leucoraja erinacea* (little skate). The Reptilia are conspicuously unsequenced, and the Amphibia are represented by *Xenopus tropicalis* (western clawed frog).

Among the simpler animal phyla, the nematode worm *C. elegans* has remarkably potent genetic tools, especially for making transgenics; it is possible to microinject embryos with plasmids that then replicate as episomal ‘rafts’, so providing effectively stable transformants overnight. By generating worms transgenic for fluorescent reporters, mutagenising them and passing them through essentially a modified FACS (fluorescence activated cell sorting) machine, it has proved possible to screen 300 000 worms in a weekend – a throughput which is the envy of the fly community (Strange, 2003; Strange, 2007). As well as several other members of the genus *Caenorhabditis*, the nematode roundworm *Trichinella spiralis* (the cause of human trichinosis) is being sequenced.

Outside of these ‘hotspots’, however, things get patchier. The echinoderm species *Strongylocentrotus purpuratus* is being sequenced, and there is an expressed sequence tag (EST) sequencing project underway for the crab *Carcinus maenus*, and a genome project for the water flea *Daphnia pulex*, the tick *Ixodes scapularis*, the hemichordate acorn worm *Saccoglossus kowalevskii*, the freshwater planarian *Schmidtea mediterranea* and the pig tapeworm *Taenia solium*. For the molluscs, sequencing of the Atlantic surf clam (*Spisula solidissima*) and *Biomphalaria glabrata* (the freshwater snail host for schistosomiasis) is underway, and the sequence for *Aplysia californica* (California sea hare) is being assembled. In the even simpler cnidarians, assembled genomic sequence is available for two sea squirts (*Cionia* spp.), and a sea anemone (*Nematostella vectensis*) and the hydrozoan polyp *Hydra magnipapillata* are being sequenced. Perhaps most exotically, the tunicate *Oikopleura dioica* and the simplest known animal (and only known member of the Placozoa), *Trichoplax adhaerens*, are being sequenced. These data are summarized in Table 2.

Of course, genomic sequence is only one criterion for integrative biology, and it must be conceded that there are no publications describing transgenic technologies for the large majority of the organisms listed above. Nor can many of the species listed above be considered to be established physiological models. So, the list of models compatible with integrative physiology in Table 1 remains fairly definitive for the time being, until the genetics catches up with the genomics.

The future for model organisms

If one considered genomic sequence and transgenic technology to be sufficient for integrative biology, then the days of the models might be limited. For example, now that genomic sequence is available for the malaria mosquito *Anopheles gambiae* and the yellow fever mosquito *Aedes aegypti*, and germ line transformation with transposon-based vectors has shown to be feasible for both, has *Drosophila* had

Table 2. *The most advanced sequencing project for a range of animal phyla*

Phylum	Species	Genome status
Vertebrates		
Mammals	*Mus musculis*	Released
Birds	*Gallus gallus*	Released
Reptiles	None	
Amphibians	*Xenopus tropicalis*	Underway
Fishes	*Danio rerio*	Underway
Invertebrates		
Arthropods	*Drosophila melanogaster*	Released
Crustacea	*Daphnia pulex*	Underway
Arachnids	*Ixodes scapularis*	Underway
Molluscs	*Aplysia californica*	Assembly
Echinoderms	*Strongylocentrotus purpuratus*	Assembly
Worms	*Caenorhabditis elegans*	Released
Tunicates	*Cionia intestinalis*	Assembly

Based on a manual search of NCBI genomes (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Genome) on 5 December 2006.

its day? Interestingly, perhaps the opposite holds true; comparative genomics approaches make it much easier to drill through to a model such as *Drosophila* and to access the extant mutant stocks and long-term research database available for the model species. The Bloomington stock centre alone holds over 20 000 stocks; whereas the technical difficulty of keeping mosquitoes means that even an active lab could not hope to maintain more than perhaps six – and then only while grant funding continued. So the ‘model organism package’ needs to be seen as a whole.

RNAi – does it promote ‘target’ species to ‘model’ status?

If the utility of mutants is accepted as a key property of a model, can RNAi be used to provide mutants in non-model species? Since its discovery, RNAi has proved a powerful experimental tool, particularly in *C. elegans*, where it suffices to feed worms on *E. coli* harbouring a plasmid encoding an RNAi hairpin construct (Wang and Barr, 2005). In *Drosophila*, there are transformation vectors available that make generation of dsRNA particularly easy (Fig. 2), and there are genome-wide screens underway both in whole flies and cell lines.

However, problems remain; RNAi relies on the subversion of a mechanism probably intended to attack invading viruses, and a common side-effect of RNAi treatment is the non-specific shutdown of transcription. Care must be taken to control also for the knockdown of closely related transcripts (so-called ‘off-target effects’). In addition, most RNAi alleles are hypomorphs, rather than nulls. This can be an advantage, as lethal mutations can be hard to study! However, we find that driving high levels of dsRNA inside cells with the GAL4/UAS system still only produces detectable knockdowns in about half of all cases (J.A.T.D., unpublished).

Given that RNAi is only partially effective when actually generated inside a cell, this might make one sceptical of the miraculous properties ascribed to RNAi in non-model organisms. dsRNA has been applied in the food, or injected into the haemocoel of larval and adult insects, and found to produce complete knockdowns that last for days, and in some cases have been heritable. These results imply that dsRNA is able to tunnel across basement membranes as well as plasma membranes, and sometimes even the blood–brain barrier. So, although RNAi (or morpholinos) will prove important in non-model species, it must be seen as a maturing technology that requires strict controls to be respected. For example, western blotting with a specific antibody to show protein knockdown, accompanied by counterstaining for a related protein that is not affected, should be seen as a minimum requirement.

Diversity and the insects

Model organisms are vital tools, but do they actually have any relevance for target species? More generally, do data in any given species have relevance to other species? This question is particularly acute for the insects, where there are perhaps as many as 30 million species in existence. Is it possible to estimate the extent to which *Drosophila* is a model insect, rather than a model fly? And is it possible to design a rational sampling strategy to cover this wide diversity with finite resources?

In the case of the *Drosophila* tubule, the evidence is promising. The importance of the apical V-ATPase is known from many species (Bertram et al., 1991; Dow et al., 1994; Garayoa et al., 1995; Maddrell and O’Donnell, 1992; Pietrantonio and Gill, 1995), and the indications that a basolateral, glibenclamide and barium-sensitive potassium channel is important are also well known (Beyenbach and Masia, 2002; Evans et al., 2005; Masia et al., 2000; Weltens et al., 1992; Wiehart et al., 2003a).

Neuroendocrine control shows great commonality, implying that neuropeptides have a common origin in insects. In all insects studied so far, cyclic AMP is diuretic and can be raised by either the corticotropin releasing factor-like DH_{44} or the calcitonin-like DH_{31} (Coast, 1998). Although the similarities with the vertebrate peptides are very faint, it could be argued that signalling through these peptides is conserved beyond insects.

The case can be made much more strongly, however, for the leucokinin family. Indeed, although the first leucokinins were characterized in an insect (Holman et al., 1984), the first gene for a leucokinin receptor, and its cognate peptide, were identified in the pond snail *Lymnaea stagnalis* in a single, thoroughly impressive paper (Cox et al., 1997). Similarly, leucokinin signalling is known to occur in the Acari (mites and ticks) (Holmes et al., 2000). In insects, leucokinins are uniformly myogenic and diuretic (Coast et al., 2002). *Drosophila* has proved useful in elucidating the mode of action of these neuropeptides; not only were the first insect leucokinin and leucokinin receptor genes identified in *Drosophila*, but the mode of action (through intracellular calcium) was established

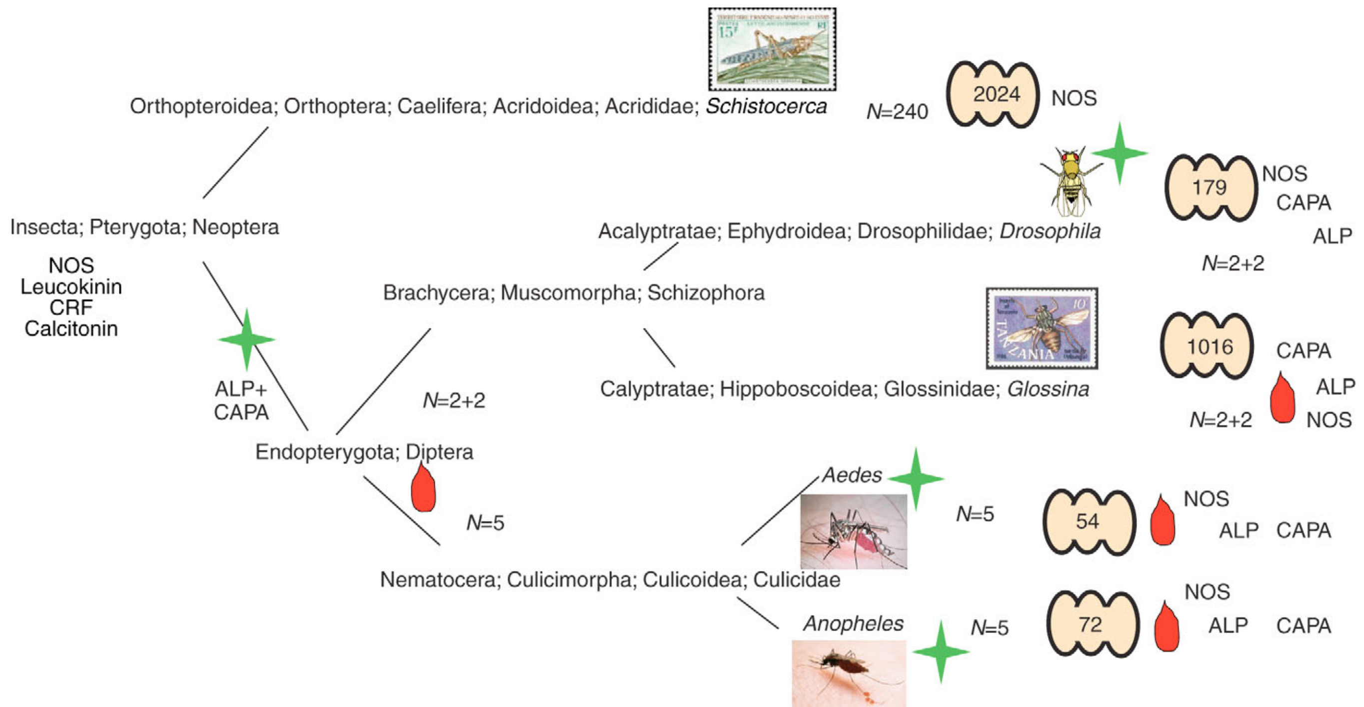

Fig. 3. Reconciling tubule physiology with phylogeny. Five species are represented; the muscomorph flies *Drosophila melanogaster* and the tsetse fly *Glossina morsitans*; the mosquitoes *Aedes aegypti* and *Anopheles gambiae*; and the more primitive orthopteran *Schistocerca gregaria*. Some recent comparative results from our lab are overlaid with classical data; the number of tubules per animal (*N*=xx), the approximate number of cells per tubule (brown shading), the presence of nitric oxide synthase in the tubule (NOS) (Pollock et al., 2004), the diuretic activity of Capa (CAPA) (Pollock et al., 2004), the existence of a defined alkaline phosphatase domain in the lower (proximal) tubule (ALP) (Cabrero et al., 2004), the presence of stellate cells (green stars) (Cabrero et al., 2004) and the haematophagous habit (red drops). The diagram shows that some properties can be considered to be common to insects whereas others seem to be associated with the Diptera. Conspicuously, tsetse flies, although closely related to *Drosophila*, lack stellate cells: parsimony suggests this is a secondary loss, perhaps associated with the degenerate lifestyle of these unusual flies.

with transgenic calcium reporter technology (Radford et al., 2002; Terhzaz et al., 1999). Leucokinin signalling is sufficiently well conserved that neuropeptides can be active across wide phylogenetic distances in the insects.

A further neuropeptide family highlights differences, as well as similarities. The Capa neuropeptides [the prototype was identified in a lepidopteran, *Manduca sexta* (Davies et al., 1994), and the first gene in *Drosophila* (Kean et al., 2002)] signal through intracellular calcium (Rosay et al., 1997). However, in *Drosophila* tubule, the cell type that receives the Capa signal is loaded with nitric oxide synthase (Davies et al., 1997), a calcium/calmodulin-sensitive enzyme. Capa peptides thus raise calcium, nitric oxide and ultimately cyclic GMP (cGMP) in the same cell (Davies et al., 1997). Both calcium and cGMP have diuretic effects in the cell; cGMP through its protein kinase (MacPherson et al., 2004), and calcium by activating mitochondria to increase the ATP supply to the apical V-ATPase (Terhzaz et al., 2006). However, although cGMP is diuretic in *Drosophila* and other Diptera, cGMP is antidiuretic in some other orders (Quinlan and O'Donnell, 1998; Wiehart et al., 2003b), and Capa can be either without effect or antidiuretic (Predel and Wegener, 2006).

So, overall, it looks as if data from *Drosophila* Malpighian tubules rolls out across all Diptera with very few caveats, and indeed the broad pattern of tubule function and control is recognizable across all insects (Fig. 3). So, the model organism/integrative physiology agenda seems to survive this relatively severe test (up to 30 million species in over 20 orders separated by 150 million years of rapid divergent evolution).

Conclusion

This review has shown that there is considerable promise in the judicious use of model organisms to establish general principles of function. It should be clear that the ease and power with which some of these organisms can be manipulated does elevate them to 'Krogh status' for several interesting biological questions. The *Drosophila* Malpighian tubule is in no way an exceptional tissue in an exotic organism; it has simply been studied in some detail. Clearly, it is vital for functional genomics that more experimental biologists take an active interest in specific tissues in these valuable organisms. This will lay the ground not just for a better understanding of what genes do but for a systems approach to the function of the whole organism. Surely this should be the ultimate goal of integrative physiology?

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