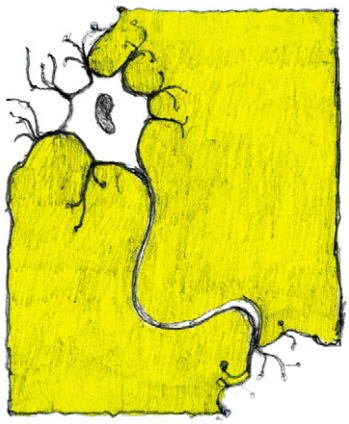
Keeping track of the literature isn't easy, so Outside JEB is a monthly feature that reports the most exciting developments in experimental biology. Short articles that have been selected and written by a team of active research scientists highlight the papers that JEB readers can't afford to miss.

Outside JEB

OLFACTORY RECEPTORS



OLFACTORY RECEPTORS TEAR UP THE TEXTBOOKS!

Ever since Linda Buck and Richard Axel's 1991 discovery that rat olfactory receptors (ORs) are seven transmembrane molecules that are part of the G-protein coupled receptor family – for which they won the Nobel Prize in 2004 – it has been generally assumed that all ORs show the same conformation.

Over the past few years, work from Leslie Vosshall's laboratory at Rockefeller University has suggested that, unlike vertebrates, insects share a common protein – Or83b – which is necessary for ORs to be integrated into the membrane. Now, in an elegant and thorough paper on *Drosophila*, Richard Benton and co-workers from Vosshall's group have not only shown how Or83b and OR proteins interact to produce a functional receptor, they have also challenged the idea that insect ORs are G-protein coupled receptors and have effectively turned the receptor molecule inside out. Their findings, if confirmed in other insects, will radically alter our understanding of the evolution and function of olfaction.

In a technical *tour de force*, the authors make use of the whole toolbox of *Drosophila* genetics. Nevertheless, the results are presented in a straightforward and clear way, with clear summaries at the end of each experiment that allow even those allergic to molecular genetics to follow the argument and the evidence.

First, Benton and co-workers showed that, in the absence of Or83b, OR molecules are unable to leave the endoplasmic reticulum. They then used the GAL80^{ts} transgene to control Or83b expression in the growing or adult fly and found that when Or83b expression was 'turned on' when the insects were 10 days old, the flies were able to begin expressing ORs in the receptor neuron membrane, and when Or83b was turned off at 3 days old, ORs gradually disappeared from the fly antenna, showing that olfactory receptors are being continually trafficked to the membrane surface throughout life.

To show that Or83b and ORs do in fact associate, the authors then made Or83b and OR transgenes fused to the N- or C-terminal fragments of a Yellow Fusion Protein, knowing that they would get a fluorescent signal if the receptors became associated. Sure enough, the receptors interacted, the molecule flouresced and further studies showed that this heteromer is functional.

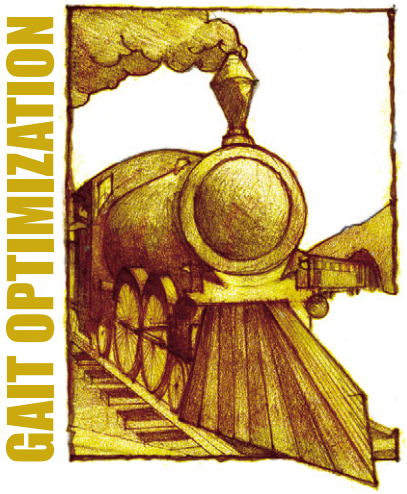
A bioinformatics study of the predicted structure of *Drosophila* ORs led to the biggest surprise: the N-terminal appeared to be intracellular, rather than extracellular as in mice. This meant that the six loops of the seven transmembrane structure changed position compared with the textbooks: the bits that were thought to be outside now seemed to be inside, and *vice versa*. The authors tested this radical suggestion in a number of ways, including a stunning immuno-electron micrograph of a horizontal section of an antennal sensillum showing 5 nm colloidal gold 'staining' of part of the OR sequence that they now predicted to be extracellular: the tiny dots were all on the outside of the dendritic membrane. The sequence and membrane topology of *Drosophila* ORs are very different to those of mammalian ORs.

Having provocatively demonstrated that *Drosophila* ORs are no more related to mouse ORs than mouse ORs are to ion channels, the authors then highlight the lack of direct evidence in the literature to demonstrate that insect ORs are in fact G-protein coupled receptors. This, coupled with the suggestion that the orientation of insect ORs is rather different to our previous understanding, will undoubtedly have ruffled some feathers amongst both scientists and textbook publishers. Buck and Axel's model has rightly become a prize-winning icon, but it might not be quite so generalised as we all thought.

10.1242/jeb.02162

Benton, R., Sasche, S., Michnick, S. M. and Vosshall, L. B. (2006). Atypical membrane topology and heteromeric function of *Drosophila* odorant receptors in vivo. *PLoS Biol.* **4**, e20.

Matthew Cobb
University of Manchester
cobb@manchester.ac.uk



OPTIMIZING WALKING AND RUNNING

With all the thousands of possible ways to move our legs, why do people prefer only two – walking and running? After all, there are many other things we could do – hopping, skipping, Monty Python silly walks, Groucho walks or the smooth level walk of a waiter with a tray full of cups of hot coffee. Most people would say that walking and running feel easiest. Other gaits are more tiring. But testing this simple and venerable idea – that walking and running use the least energy at low and high speeds, respectively – is surprisingly difficult to do.

Rather than trying to measure energy consumption of people walking strangely, Manoj Srinivasan and Andy Ruina built a minimal mathematical model of bipedal locomotion, which they describe in *Nature*. Their goal was to simplify locomotion enough that, for a given speed and step length, they could choose the optimal energy-conserving gait out of the infinite space of possible periodic motions.

The model approximates each leg to something like a reverse pogo stick. Rather than absorbing energy through a spring, each leg can lengthen up to the maximum leg length, applying a force to the ground. Between steps, the body can become aerial, only being affected by gravity. Only one leg can contact the ground at a time. With these simplifications, the researchers used standard optimization techniques to find the forces each leg would have to apply to the ground to produce the most cost-efficient locomotion at a given speed and stride length.

Despite the fact that legs aren't reverse pogo sticks, the model found two optimal gaits that look a lot like walking and running – and one gait that humans don't use. At low speeds and low stride lengths, the model produces an 'inverse-pendulum' walk, in which the body vaults over a stiff leg much like our own walking gait. The leg produces forces only for very short periods at the beginning and end of the step. At high speeds, the model settles into an impulsive run, in which the body flies through the air most of the time and the leg only touches down very briefly before launching the body again. In between these two caricatures of walking and running, the model found a hybrid gait: at low speeds, but long stride lengths, the reverse pogo stick uses an inverse-pendulum step, like a walk, but with an aerial phase like a run. The researchers speculated that where healthy, strong people might normally change from walking straight to running, weak or obese people might choose something like the hybrid gait because of an inability to maintain true running speeds.

Srinivasan and Ruina also found a fairly simple explanation for why walking and running are optimal gaits. They have a key similarity: they both compress all force production (and thus energy expenditure) into very short impulses – for walking, at the very beginning and end of the step, and for running, the brief foot contact period. The rest of the cycle – for walking, the inverse pendulum period, and for running, the aerial period – requires no energy. Other gaits, like a flat level walk, require energy throughout the stance phase and are thus less efficient. They really are more tiring.

10.1242/jeb.02158

Srinivasan, M. and Ruina, A. (2006). Computer optimization of a minimal biped model discovers walking and running. *Nature* **439**, 72-75.

Eric Tytell
University of Maryland
tytell@umd.edu

FROZEN INSECTS STARVING FOR OXYGEN

Freeze-challenged insects can invoke either of two strategies. They may resist freezing altogether, using an arsenal of molecules that inhibit ice formation; good freeze-avoiders remain liquid to below –30°C. Alternatively, insects may allow or even encourage ice formation, but with strict conditions on when and where crystals form. Unexpectedly, however, the obvious threat to freeze-tolerant insects – cell damage from ice crystals – is not the only threat. They must also cope with oxygen starvation. Oxygen moves at glacial speeds through ice at the tips of tracheoles. Moreover, tracheal ventilation usually depends on movement, which is impossible if muscles are partially frozen. How do frozen insects withstand hypoxia?

Pier Morin, David McMullen and Ken Storey examined this problem using the famous freeze-tolerant fly, *Eurosta solidaginis*. Larval *Eurosta* stimulate gall formation in goldenrods, and they live and feed in their galls throughout the summer. Full-grown larvae also overwinter in their galls – a strategy fraught with peril, as galls often are not insulated by snow and can reach very low temperatures. A mid-winter *Eurosta* may be mostly frozen.

The team focused on *Eurosta*'s hypoxia-inducible factor 1α (HIF-1α). The molecule is known from studies of mammals (and a few insects and other invertebrates) to play a key role in triggering and coordinating gene expression in response to hypoxia. In short, high oxygen levels promote HIF-1α degradation, whereas low oxygen levels allow persistence. If it persists, it binds to another (constitutively expressed) molecule, HIF-1β, and the complex moves into the nucleus. There it acts as a transcription factor activating genes

involved in the hypoxia response by binding to a conserved sequence known as the hypoxia response element.

The team quantified levels of *hif-1α* transcripts in larvae exposed to either anoxia or cold to see how the transcription factor responded to hypoxia and cold conditions. In the anoxia experiments, where the team subjected larvae to pure nitrogen for a day, the insects showed a 2.8-fold rise in transcript levels; and larvae given an extra day to recover had higher levels still. Looking at the larvae's responses to cold exposure, the team found that a chill also stimulated *hif-1α* transcript levels. Larvae cooled to 3°C for a day showed a 1.7-fold rise in *hif-1α* levels, as did those subsequently frozen for a day at –16°C. The team also examined expression levels of HIF-1α protein, and these too were higher in 3°C larvae.

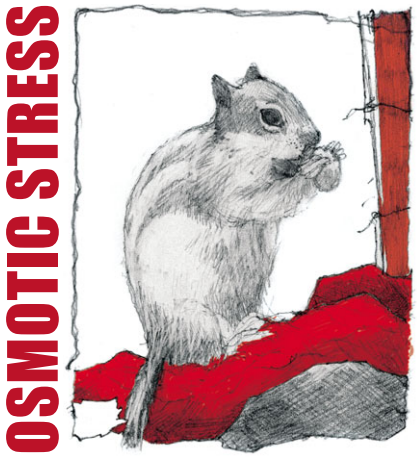
But larvae at 3°C were probably not oxygen-limited, as metabolic rates would have been low and tracheal systems unobstructed by ice. So why had their *hif-1α* levels risen? The authors suggest that chill-induced expression *anticipates* freeze-induced hypoxia, providing just-in-time preparation for the metabolic disruption of oxygen deprivation.

When *Eurosta* are cold, the master switch clearly sHIFts from off to on. What remains is to elucidate downstream consequences for gene expression and, ultimately, for the metabolic phenotype.

10.1242/jeb.02157

Morin, P., Jr, McMullen, D. C. and Storey, K. B. (2005). HIF-1α involvement in low temperature and anoxia survival by a freeze tolerant insect. *Mol. Cell. Biochem.* **280**, 99-106.

Art Woods
University of Texas at Austin
art.woods@mail.utexas.edu



HENLE'S LOOP AND THE OSMOTIC STRESS PROTEOME

Cells of the thick ascending limb of Henle's loop (TALH) in the human kidney are exposed to extreme hyperosmotic stress during urine formation. They are actively involved in transporting salts (mostly NaCl) from the renal tube into the extracellular space of the medulla. However, because the ascending limb of the loop is impermeable to water, the accumulation of ions in the cells creates extreme hyperosmotic conditions. Such cellular conditions are known to trigger an increase in sorbitol due to the upregulation of aldose reductase, an enzyme that directs glucose towards sorbitol. In addition, several heat-shock proteins have also been found previously to be upregulated under osmotic stress in cells of the TALH. Knowing that extreme osmotic conditions can trigger such physiological changes, it would be intriguing to take a bird's-eye view of these cells to see other changes in protein expression that characterize their response to one of the most, if not the most, severe stresses that human cells can be exposed to. Hassan Dihazi and colleagues from the Georg-August University of Göttingen in Germany used a proteomic approach to obtain this kind of global view to gain new insights and make some very interesting discoveries.

The authors used two-dimensional gel electrophoresis to investigate the differences in protein expression in an epithelial cell line from the outer medulla of a rabbit, which has been shown to be a suitable model for the function of TALH cells. They exposed cells to normal (300 mosmol kg^{-1}) and hyperosmotic (600 mosmol kg^{-1}) stress conditions. After comparing expression levels, they isolated protein spots from the gels, digested them with trypsin and analyzed the mass of the resulting peptide fragments. The distribution of the masses of these fragments that one can obtain with mass spectrometry provides a so-called 'mass fingerprint'. Comparing the mass fingerprints with the genome, the team found 25 proteins to be overexpressed and 15 downregulated in the cells when exposed to hyperosmotic stress. What did they learn?

Importantly, the 15-fold upregulation they found in aldolase reductase levels confirmed an expected finding and validated the approach taken. In support of the importance of sorbitol accumulation, the authors also observed the upregulation of lactate and malate dehydrogenase, enzymes that participate in gluconeogenesis and provide increased levels of glucose, which is needed for the synthesis of sorbitol.

Hyperosmotic stress causes cell shrinking and therefore requires changes in the expression of cytoskeletal proteins. Vimentin and tropomyosin, two proteins that are associated with the cytoskeleton, changed expression levels, which confirmed the importance of cell-volume modifications during hyperosmotic stress.

The authors also compared the response of their control cell line with cells that were selected after exhibiting increased resistance to higher osmotic conditions (600 mosmol kg^{-1}), under which they were still able to grow. The resistant cells showed higher expression levels of several heat-shock proteins, such as α-crystalline, Hsp70 and Hsp90, which have several functions including the maintenance of protein structure during stress. Surprisingly, other stress proteins, such as glucose-regulated proteins that are located in the endoplasmic reticulum and are involved in Ca^{2+}-binding, were downregulated, which the authors suggest may augment Ca^{2+} homeostasis, which plays an important yet still rather unknown role during osmotic stress.

The diversity of cellular responses that such a proteomic approach reveals is unsettling, reminding us how little we actually know about how the cells in the human kidney respond when they are exposed to one of the most severe stressors that human cells experience. In many ways, this bird's-eye view has raised more questions than it answered.

10.1242/jeb.02160

Dihazi, H., Asif, A. R., Nitin, K., Doncheva, Y. and Müller, G. A. (2005). Proteomic analysis of cellular response to osmotic stress in thick ascending limb of Henle's loop (TALH) cells. *Mol. Cell. Proteomics* **4**, 1445-1458.

Lars Tomanek
California Polytechnic State University
San Luis Obispo
ltomanek@calpoly.edu

CLIP IT

It's a well-known fact that proteases facilitate digestion. However, nature has also evolved proteases whose primary function is not degradation, but specific cleavage of proteins to activate their functions. Frequently, the targets are themselves proteases that cleave other ones. This yields cascades of successive proteolytic reactions that rapidly amplify those signals that initiated the first cleaving step. Such proteolytic cascades are used in signalling and regulate important physiological functions.

Insects have perfected a network of extracellular serine proteases that control embryonic development and both blood clotting and the innate immune response. Many of the proteases involved in these functions belong to the family of Clip-domain serine proteases that have one or two so-called Clip-domains attached before the serine protease domain. The early stages of melanization, one component of the innate immune system, are catalyzed by a phenoloxidase, which is produced as an inactive precursor, the prophenoloxidase. Prophenoloxidase is activated by prophenoloxidase-activating factors (ppAFs), However, as part of the signalling cascade, ppAFs are also synthesised as pro-proteins that require activation before they can in turn activate prophenoloxidase. The ppAFs include Clip-domain serine proteases (ppAF-I/III), and Clip-domain serine protease homologues (ppAF-II), which contain a serine protease domain that has lost catalytic activity.

To uncover the functions of Clip-domains, a Korean–Japanese team led by Bok Leul Lee, Byung-Ha Oh and Nam-Chul Ha have crystallized the inactive Clip-domain serine protease homologue ppAF-II from the beetle *Holotrichia diomphalia* and determined the protein's structure. Analysing the structure of the Clip-domain yielded a novel protein fold with a central four-stranded, irregular β-sheet entwined by numerous loop-like structures that are interlinked by three disulfide bonds. The Clip-domain is tightly connected to the serine protease homologue domain and contains a central cleft that could bind hydrophobic protein regions or other hydrophobic patches.

As ppAFs are activated by upstream proteases, the team asked whether cleavage and activation changes the proteins' quaternary structure. After cleaving ppAF-II with a recombinant upstream protease and analysing its structure by electron microscopy, they found that the activated ppAF-II is no longer a monomer but a dodecamer composed of two hexameric rings. The additional finding that the activated ppAF-II can interact with phenoloxidases thereby forming supramolecular, ball-like structures is interesting as this would allow phenoloxidases to cluster, facilitating subsequent enzyme reactions. To map ppAF-II's phenoloxidase binding site, the scientists produced recombinant ppAF-IIs with altered amino acids and tested them for phenoloxidase binding and oligomerization. It turned out that the central cleft of the Clip-domain binds the phenoloxidase but does not mediate ppAF oligomerization.

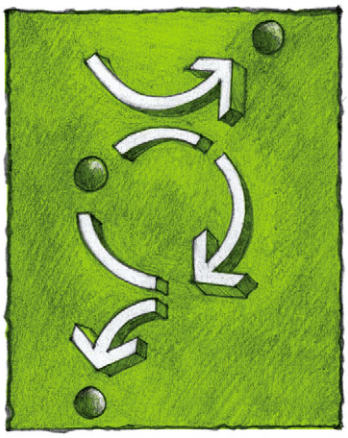
Wondering how the Clip-domain functions in ppAFs that retain a serine protease activity, the team analysed the properties of ppAF-I and found that the Clip-domains are not tightly associated with the protein's serine protease domain, possibly allowing the Clip-domain to bind to the surfaces of invading pathogens, leaving the serine protease exposed to interact with other components of the signalling cascade. This step could be crucial for the local amplification of signals that initialise the immune response, suggesting that Clip-domain serine proteases serve multiple functions in insect innate immunity and beyond. Both ppAFs, with and without serine protease activity, evidently play different roles in melanization. What we learn from this system is that slight variations in the arrangement of different protein domains allows diversification and tuning of regulatory mechanisms, a process that may be an essential building block in an insect's survival strategy.

10.1242/jeb.02159

Piao, S., Song, Y.-L., Kim, J. H., Sam, Y. P., Park, S. Y., Park, J. W., Lee, B. L., Oh, B.-H. and Ha, N.-C. (2005). Crystal structure of a clip-domain serine protease and functional roles of the clip domains. *EMBO J.* **24**, 4404-4414.

Hans Merzendorfer
University of Osnabrueck
merzendorfer@biologie.uni-osnabrueck.de

AROUSAL PROTECTION



AROUSED HAMSTERS SCAVENGE REACTIVE OXYGEN SPECIES

While a great deal of research has focused on the induction of hibernation in mammals and how they are able to survive long periods with a drastically reduced metabolic rate and blood flow, recent research is beginning to focus on the other end of the equation, asking, 'How do hibernating animals survive reoxygenation and increased blood flow as they increase metabolic rates back to normal?' Oxygen consumption and respiratory rates in hibernators may increase to as much as 300% of resting cenothermic (normal body temperature) levels during the shivering stage of arousal thermogenesis. In most animals, a sudden increase in blood flow or increase in oxygen supply, after a period of low perfusion, results in the production of highly reactive oxygen (ROS) and reactive nitrogen species. When ROS levels exceed cellular antioxidant capacity, tissue injury results as the high-energy compounds interact with DNA, proteins and lipids.

Hibernating ground squirrels have been shown to increase the activities of antioxidant enzymes and plasma ascorbate during hibernation, presumably in preparation for the intense metabolic activity of arousal and large fluxes in blood flow and temperature. The recent study by Peter Osborne and Masaaki Hashimoto in *Behavioral Brain Research* extends these findings by profiling concentrations of the antioxidants ascorbate, glutathione and urate in the hamster brain during hibernation, arousal and between hibernation bouts. Using very slow flow microdialysis, in which a probe with a semi-permeable membrane tip is inserted into the striatum (an area of the brain rich with the neurotransmitters glutamate and dopamine, and therefore highly vulnerable to oxidative damage) and perfused with artificial cerebral spinal fluid, they were able to measure changes in the brain extracellular fluid independent of temperature. Total brain tissue and plasma antioxidant levels were also recorded.

They found that brain tissue levels, striatal extracellular (ECF) concentrations, and plasma levels of the antioxidants are differentially regulated during hibernation and during the metabolically demanding transition from hibernation to cenothermia. Surprisingly, mid- and forebrain tissue levels of ascorbate did not change from hibernation to cenothermia, even though plasma and ECF levels varied significantly. Plasma and ECF ascorbate increased significantly during hibernation (to more than 400% over cenothermia) and then decreased upon arousal, presumably because of the scavenging of free radicals generated during the reperfusion period. The authors hypothesize that ascorbate increased in the ECF, but not the whole tissue, because uptake into the cells decreased while uptake from the plasma into the ECF continued. ECF glutathione concentrations were low during hibernation and increased during arousal and cenothermia, while levels remained fairly constant under each condition in tissues and plasma.

By contrast, ECF urate levels were regulated at a constant level during hibernation and cenothermia (with a transient doubling of ECF levels during shivering thermogenesis), despite a 50% reduction in intracellular concentrations during hibernation. Because urate, along with superoxide and hydrogen peroxide, is produced as a metabolite of xanthine activity, this increase can be viewed as a marker of ROS production as well as a means of ROS defence.

Looking at work done on several different species, the authors point out that the only common pattern of arousal metabolism is significant oxidation of ascorbate from the extracellular and plasma spaces, while brain tissue content of ascorbate and glutathione remains constant; whether this is because the brain is insulated from extracellular changes or is able to tightly regulate antioxidant levels is yet unknown. Very slow flow microdialysis, then, allows a real-time look at the metabolically demanding events of hibernation and arousal, which may provide investigators with a way to address these questions. And by looking at animals adapted for metabolic depression and arousal, it may be possible to separate protective regulatory mechanisms from the background pathological events observed in other mammalian brains.

10.1242/jeb.02161

Osborne, P. G. and Hashimoto, M. (2005). Brain antioxidant levels in hamsters during hibernation, arousal, and cenothermia. *Behavioral Brain Res.* doi:10.1016/j.bbr.2005.11.007.

Sarah L. Milton
Florida Atlantic University
smilton@fau.edu