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REVIEW

Gene expression, metabolic regulation and stress tolerance during diapause

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Abstract Diapause entails molecular, physiological and morphological remodeling of living animals, culminating in a dormant state characterized by enhanced stress tolerance. Molecular mechanisms driving diapause resemble those responsible for biochemical processes in proliferating cells and include transcriptional, post-transcriptional and post-translational processes. The results are directed gene expression, differential mRNA and protein accumulation and protein modifications, including those that occur in response to changes in cellular redox potential. Biochemical pathways switch, metabolic products change and energy production is adjusted. Changes to biosynthetic activities result for example in the synthesis of molecular chaperones, late embryogenesis abundant (LEA) proteins and protective coverings, all contributing to stress tolerance. The purpose of this review is to consider regulatory and mechanistic strategies that are potentially key to metabolic control and stress tolerance during diapause, while remembering that organisms undergoing diapause are as diverse as the processes itself. Some of the parameters described have well-established roles in diapause, whereas the evidence for others is cursory.

Keywords Diapause · Gene expression · AMP-activated protein kinase · Molecular chaperone · Stress tolerance · Reactive oxygen/nitrogen species · Apoptosis · LEA proteins

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Introduction

Diapause, a developmental program that has existed in metazoans for millions of years [1], is characterized by processes opposite to those of reproductive growth, such as the arrest or slowing of cell division in response to an immediate or anticipated stress, reduction of metabolism and enhancement of stress tolerance, at times to high levels [2–8]. The process of diapause occurs at different genetically established developmental stages, but usually at only one life history stage in any given organism, and it provides adaptive benefits. Two favorable outcomes of diapause are survival of environmental extremes and the synchronization of reproductive activities. Moreover, the reactivation and development of organisms that have lain dormant for periods of time that can extend into years mix temporally separated genotypes, possibly to advantage in ways that would not otherwise occur [2, 9].

Diapause is divided into overlapping stages encompassing induction, preparation, initiation, maintenance and termination, with the latter followed by post-diapause quiescence if environmental conditions are not conducive to growth [3, 6]. The entry into and progression through diapause are mediated by molecular mechanisms similar to those that guide cellular reproductive growth; these include differential gene expression, post-transcriptional events, post-translational protein modifications and protein localization to specific regions within cells and organisms. The ability to execute these events in a coordinated manner implies the function of molecular switches that exert regulatory roles, directing development along the appropriate pathways. Identifying the switches and the extent to which they are shared between organisms are central questions in the study of diapause. Although answers remain elusive, it is known that genes characteristic of reproductive growth

are down-regulated during diapause, while other genes, often termed diapause-specific, are up-regulated, and the expression of still others is either not altered or changes intermittently [3, 10–14]. Gene expression and progress through diapause are likely to be influenced by signaling pathways, many of which are receptor responsive and perceive extracellular factors, whether generated by other cells or as a physical signal originating in the environment, the best studied being for *Caenorhabditis* and *Drosophila* [8, 15–17]. Internally derived signals including reactive oxygen/nitrogen species (ROS/RNS) like hydrogen peroxide (H_2O_2) and nitric oxide (NO) may regulate diapause-specific developmental pathways. H_2O_2 and NO modulate development and physiological activity in many organisms, often in a coordinated manner [18–20], and they support the germination of plant seeds [21, 22], biological structures similar to diapause-specific resting stages of organisms like *Artemia franciscana* [23].

Arising from the regulatory processes mentioned above are changes from one type of intermediary metabolism to another, metabolic rate depression and the diversion of energy expenditure away from cellular events required for an active lifestyle [7, 10, 24, 25]. For example, energy saved by inhibition of translation and ion pumping, both controlled in part by reversible protein phosphorylation [24, 26, 27], becomes available for the construction of morphological structures such as protective coverings that prevent organismal rupture, collapse during stress and water loss [8, 28, 29]. The synthesis during diapause of molecular chaperones able to inhibit irreversible protein denaturation and apoptosis [3, 4, 10, 23, 30, 31] is empowered by redirection of energy reserves, as is the production of late embryogenesis abundant (LEA) proteins that protect against dehydration [32–37]. Sugars such as trehalose, synthesized in some diapause-destined organisms and thought to act in concert with proteins, shield against drying damage [34].

The broad range of mechanistic processes displayed by diapausing organisms, where genes are expressed and energy is utilized to produce properties different from those in growing cells, converges to common outcomes. Respiratory and intermediary metabolism adjust to environmental conditions and the generation of molecules required for survival during diapause, the cell cycle reversibly arrests, and stress tolerance is enhanced to protect cell attributes required for resumption of growth and development when diapause terminates. However, the multiplicity of organisms capable of undergoing diapause and the diversity of developmental programs recognized as diapause dictate that the properties of this developmental pathway will vary from species to species. By way of example, diapause occurs in flat worms where it is known as dauer [8, 10, 15], crustaceans [2, 9, 23, 38, 39], rotifers [14], tardigrades [40–42], insects [3, 6, 7, 13, 16, 17], killifish [43, 44] and mammals, the latter as delayed embryo implantation [4]. In addition, embryonic diapause has been reported for *Caenorhabditis elegans* [45]. Within these groups diapause manifests in different developmental stages including embryos, pupae, larvae or adults, thus adding to complexity. The degree of dormancy ranges from cessation of development and very low metabolism in the embryonic diapause of *A. franciscana* [23, 46, 47] and the annual killifish, *Austrofundulus limnaeus* [26, 48], to relatively mild dormancy where metabolism and growth continue at a lower rate than during reproductive metabolism, as in the larval and adult diapauses of some copepods [9] and insects [3, 7, 13, 16]. The level of stress tolerance achieved during diapause varies, along with the degrees of morphogenesis and behavioral modifications contributing to survival under adverse conditions [8, 16, 23]. The nature and timing of external signals such as day length or temperature, acting alone or in concert, that induce diapause via neurohormonal mechanisms in many organisms differ, as do cues that end dormancy, which in *C. elegans* requires only return to favorable growth conditions [2, 3, 6, 8, 17]. It is of interest, therefore, to consider diapause properties—those grounded in fact as well as those of a speculative nature—and to identify cell/molecular commonalities that exist in spite of differences at higher organizational planes (Fig. 1). This is done throughout the review with emphasis on metabolic regulation and stress tolerance rather than the equally interesting processes of diapause initiation and termination per se.

Gene expression during diapause

Differential gene expression during diapause is scrutinized in numerous studies by homology-based identification of mRNAs and proteins on a one-by-one basis. This approach is complemented by the establishment of expressed sequence tag (EST) libraries [49–52] and the application of methodologies permitting multiple-gene analysis in single experiments such as proteomics [12, 39, 53], suppressive subtractive hybridization [11, 14, 38, 54] and microarray analysis [13, 55], these often coupled with PCR-based quantification and probing of northern blots. The result is an expanding list of mRNAs and proteins that increase, decrease or are unchanged during diapause. Some of these approaches, although informative, are limited because only mRNA is examined, and others, although showing the presence of particular proteins, fall short of demonstrating function. Mutational studies, gene knock outs and protein knock down by RNA interference (RNAi) are required to confirm protein functionality. Despite the limitations it is nonetheless very instructive to consider data generated by multi-gene analysis because they comment on earlier studies and suggest avenues for subsequent investigations.

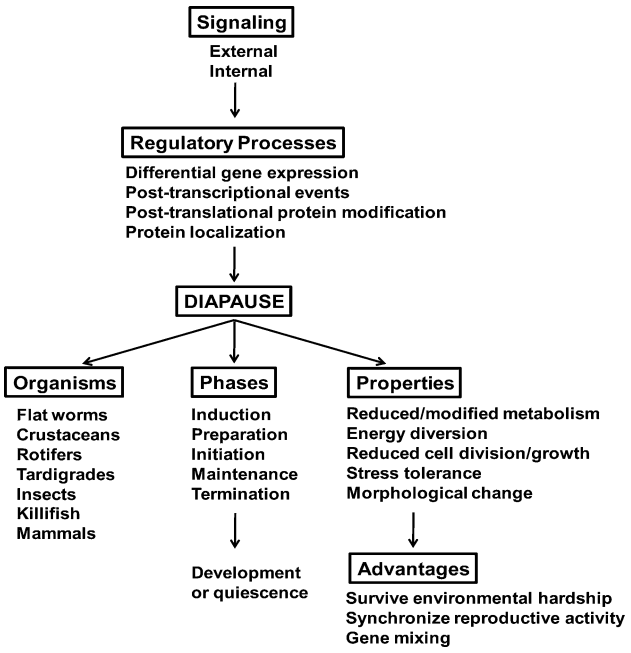

Fig. 1 *Diapause*. Regulatory processes triggered by external and internal signals occur in many organisms capable of undergoing diapause, a physiological process characterized by overlapping stages. All diapause organisms exhibit one or more properties, in varying degrees of intensity, which enable survival upon exposure to harsh environments and permit the mixing of genes from temporally separated populations. This review demonstrates underlying similarities between diapauses in organisms that may otherwise appear unrelated and that diapause in different organism is often characterized by shared properties expressed in varying intensities

Microarray technology and immunofluorescence were used to explore delayed implantation of mouse embryos [55, 56]. Delayed implantation is a hormone-controlled state of suspended animation where blastocyst cell proliferation and metabolism cease, thereby precluding embryo implantation. Six categories of genes including those involved in cell cycle regulation, metabolism, intracellular signaling, transport into the nucleus, chromatin remodeling and cell adhesion display altered expression upon comparison of diapausing and active mouse embryos. Delayed implantation has similarities to *C. elegans* dauer, but it potentially differs in the degree of stress tolerance because molecular chaperones are not up-regulated in dormant mouse blastocysts, whereas sHSPs accrue during dauer [57]. The difference reflects the more variable environment experienced by *C. elegans* as compared to the blastocyst inside an animal.

When preparing for diapause, a process initiated by juvenile hormone reduction, adult females of the northern house mosquito, *Culex pipiens* L. enhance lipid content by feeding on carbohydrate-rich plant secretions rather than blood [54]. Suppressive subtractive hybridization and probing of northern blots reveal differential regulation of eight gene categories at various stages of *C. pipiens* diapause. There are similarities to the types of genes expressed in mammalian embryos experiencing delayed implantation such as those modulating metabolism, food use and cell growth regulation. One difference is the slight up-regulation of genes encoding a sHSP and *Hsp70*, perhaps required for overwintering protection, although neither molecular chaperone is considered a major contributor to *C. pipiens* diapause. By comparison, stress proteins play an important role in pupal diapause of the flesh fly *Sarcophaga crassipalpis* [53, 54, 58–60]. Among other stress responses in *C. pipiens*, a gene encoding the antioxidant selenoprotein, a protein with the capacity to influence lipid peroxidation, is up-regulated. This observation highlights the need to control the redox state of diapause organisms, both as a protective mechanism and in cell signaling.

Drosophila melanogaster lack a strong diapause response; nonetheless, oogenesis is impeded at pre-vitellogenesis if reproduction is unlikely to be successful, leading to a physiological state termed reproductive diapause [13]. Arrested eggs resume development after several weeks of dormancy or when females return to permissive conditions. Although giving rise to a superficial or light dormancy, reproductive diapause is under hormonal and circadian regulation. The analysis of *Drosophila* reproductive diapause is complicated by the concurrent expression of genes engaged in early egg development. To facilitate categorization, Baker and Russell [13] proposed that diapause-specific genes are up-regulated within 24 h of diapause induction in *Drosophila*, and their expression does not change significantly until oogenesis resumption. Genes that change expression when post-diapause vitellogenesis begins may also mediate reproductive diapause. Employing these guiding criteria, microarray analyses established the differential regulation of genes engaged in RNA and protein production during *Drosophila* reproductive diapause, with TOR signaling pathway members prominent. *Hsp23* and *Hsp70* mRNA increase, and their protein products could protect proteins from stress-induced irreversible unfolding. Juvenile hormone catabolism is enhanced, perhaps potentiating diapause entry, a process requiring mediation of growth suppression and lipid storage by the insulin signaling pathway. Enzymes of lipid and carbohydrate metabolism are prominent although their activity declines as diapause progresses and animals prepare to over-winter. The buildup of proteolytic enzymes is likely to pave the way for resumption of digestion upon diapause termination. Cuticle structural components are enriched, as are chitin-binding proteins, changes with the capacity to decrease cuticle permeability, prevent water loss and promote survival [13].

Responding to external and internal factors, the parasitic solitary wasp *Nasonia vitripennis* undergoes maternally induced developmental diapause, with the last larval instar entering dormancy prior to pupation [12]. Non-gel shotgun proteomics revealed the exclusive synthesis of only a few proteins during early *Nasonia* diapause, but differences were observed in the abundance of proteins concerned with fat and sugar metabolism, results reminiscent of diapause in other invertebrates. The accrual of macromolecules required for protein synthesis and degradation switched, as diapause progressed, to the accumulation of proteins engaged in maintenance. For example, elongation factor 1 and an assortment of ribosomal proteins declined, corroborating reports of protein synthesis and cell growth regression as larvae enter diapause. Reduced histone levels correlate with falling protein synthesis and cell replication, events possibly related to increases in actin and fluctuating proteolytic enzymes, both associated with cell restructuring. Actin changes in *C. pipiens* were suggested to modulate the cytoskeleton early in diapause [61]. Metabolism adjusts to maintain diapause as cell growth decreases, and in *Nasonia* this includes the synthesis of glycerol, a cryo-protective molecule. Other protective proteins augmented during *Nasonia* diapause are ferritin, which may shield against oxidative damage, hinder cell growth by sequestering iron or perform both actions. In addition, Hsp90 dwindles as diapause progresses, while Hsp20, likely to chaperone denaturing proteins, increases.

Subtractive hybridization [11] and proteomics [39] were used respectively to study diapause in the crustaceans *A. franciscana* and *A. sinica*. Diapausing *Artemia* embryos enter a profound state of dormancy, and they possess exceptional stress tolerance. At 2 days post-fertilization, the quantities of several mRNAs expand in diapause-destined embryos, but not in embryos developing directly into larvae [11]. This group of mRNAs, whose accumulation was verified by RT–PCR, accommodates a stress-responsive transcription co-factor termed p8, homologues of human tumor suppressor proteins with potential as cell cycle inhibitors, sHSPs and several enzymes, such as glucose-6-phosphate isomerase, cytochrome P450 and chitin-binding proteins, their presence disclosing alterations in cell metabolism. Diapause *A. sinica* cysts were examined by proteomics-based methodology, but comparisons were not made to other life history stages. A sHSP equivalent to *A. franciscana* p26 was the major spot on two-dimensional gels containing protein extracts of *A. sinica* cysts and Hsp70 was present, both presumably required for stress tolerance. Several enzymes responsible for carbohydrate and energy metabolism were identified, and they were proposed to have a role in the resumption of development. Additionally, mediators of protein synthesis like Hsp70, a tRNA synthetase, proteolytic enzymes and cell cycle regulators increased in *A. sinica* diapause cysts. It is difficult to compare the studies done with *Artemia* because different methodologies were employed and the embryos were examined at dissimilar stages, but they do open a window on proteins synthesized in organisms experiencing profound dormancy during diapause.

The rotifer *Brachionus plicatilis*, a zooplanktonic invertebrate, produces resting or diapause eggs that persist for decades and permit survival even though the organism inhabits ephemeral bodies of water [14]. Subtractive hybridization revealed several up-regulated dormancy-associated *Brachionus* mRNAs with the capacity to protect intracellular macromolecules. mRNAs encoding proteins implicated in anti-oxidant activity and detoxification are highly represented in *Brachionus* EST libraries. These consist of glutathione *S* transferases, known to be associated with *C. elegans* dauer, dismutases that catalyze the formation of H_2O_2 from superoxide radicals, peroxiredoxins and thioredoxin peroxidase. Hsp70 and sHSPs are found, with the latter highly represented in resting eggs of *Brachionus*, as is true for *Artemia* cysts. The molecular chaperones undoubtedly confer macromolecular protection, preventing irreversible stress-induced protein denaturation and contributing to refolding when development resumes. *Brachionus* synthesizes Group 3 LEA proteins, first found in plants where they enhance resistance to desiccation and stress, and since then observed in *Artemia* and *C. elegans*. LEA proteins are discussed in more detail later in the review. Metabolic parameters were considered with interest in genes required for the synthesis of lipids, potentially a major energy source. Several lipoprotein lipase transcripts were noted, and this enzyme may replace vitellogenin in yolk platelets.

Methodologies permitting multiple-gene analysis prove diapause is characterized by the regulation of a plethora of genes varying from species to species. Common themes emerge, frequently echoing those generated by homology-based identification of mRNAs and proteins. Many questions are posed by these findings, and several will be considered, beginning with a speculative consideration of metabolic rate depression, a fundamental diapause characteristic.

Does AMP-activated protein kinase (AMPK) mediate diapause-induced metabolic rate depression and dormancy?

Survival time during diapause and quiescence tends to be proportional to the extent of metabolic depression, making the regulation of metabolic reduction, often accompanied by an increased AMP:ATP ratio, critical to continued existence during dormancy [62–64]. Sensitivity to the intracellular AMP:ATP ratio or energy charge, and a key to

control of cell growth and division in response to extracellular signals, is the liver kinase B1-AMP-activated protein kinase (LKB1-AMPK) pathway [65–68]. AMPK was discovered in mammalian cell protein extracts where it functioned as an antagonist of lipid biosynthesis by phosphorylating key enzymes responsible for the manufacture of cholesterol and fatty acids. AMPK is a hetero-trimer composed of the catalytic α-monomer and the regulatory subunits β and γ, products of different, often multi-copy genes. The α-subunits, recognized as serine/threonine kinases, have a highly conserved threonine residue phosphorylated by the up-stream kinase LKB1, a functional tumor suppressor when bound to co-factors STRAD and MO25 [68]. Contrary to what might be expected, LKB1 is not activated directly by AMP even though the AMP:ATP ratio increases in metabolically stressed cells. Instead, AMP binds to Bateman domains in the regulatory γ-subunit, promoting net phosphorylation of the α-subunit by reducing its effectiveness as a phosphatase substrate. The phosphorylated α-subunit is also stimulated allosterically by AMP, which acts synergistically with increased phosphorylation to greatly enhance AMPK, a process requiring only a minor increase in nucleotide concentration. Additionally, the β-subunit has a glycogen-binding domain, potentially allowing response to glycogen levels, an interesting possibility in the context of energy sensing that has yet to be investigated thoroughly [69].

AMPK is triggered by hypoxia and other metabolic stresses that interfere with ATP synthesis. Under energy-limiting circumstances activated AMPK has the capacity to turn on alternative ATP producing catabolic pathways ensuring sufficient energy is available to sustain organisms as overall energy production wanes, a common situation during diapause. Furthermore, as diapause prevails, major energy utilizers like protein synthesis decline upon inhibition of both initiation and elongation [70, 71]. Protein synthesis initiation is limited in some cells by the tumor suppressor tuberous sclerosis complex (TSC2), a target of AMPK. TSC2 decreases phosphorylation of ribosomal S6 kinase (S6 K) and eukaryotic initiation factor 4E binding protein 1 (4EBP1), important regulators of protein synthesis that function via the target of rapamycin (TOR) pathway [70]. In addition, protein synthesis is inhibited by a TOR-independent mechanism where AMPK activates eukaryotic elongation factor 2 (eEF2) kinase, leading to eEF2 phosphorylation and nullification of its activity [71]. Thus, by impeding protein synthesis AMPK eliminates a major cellular energy demand and curbs hypertrophy of non-dividing cells, both effects aiding survival by coordinating energy levels and growth [68, 70]. As just described, AMPK exerts acute regulation within cells by phosphorylating metabolic enzymes and protein synthesis factors. Longer term control is exercised through phosphorylation of p300, a transcriptional co-activator able to modulate the expression of genes participating in metabolic pathways influenced by AMPK [72], or by a combination of transcriptional and post-translational mechanisms acting on discrete cellular processes [68]. For example, AMPK reduces apoptosis induced by energy stress; this is important for organisms undergoing diapause [73].

The potential significance of AMPK-dependent regulatory mechanisms in organisms experiencing reduced metabolism and development due to diapause is obvious, but is there evident for this? *Artemia* undergo profound metabolic depression during diapause [46, 47, 74], but respiration rates for mitochondria isolated from diapause and metabolically active embryos are similar [74]. Apparently, changes to mitochondria do not explain metabolic rate depression in diapause *Artemia* embryos, and this leaves the way open for other mechanisms including those mediated by AMPK. *Artemia* contain AMPK, although it appears not to be phosphorylated in early post-diapause embryos when the AMP:ATP ratio is highest [75, 76]. Phosphorylated AMPK is first detected 4 h post-diapause and increases for 12 h, but it is absent from nauplii and adults. Delegation of phosphorylated (active) AMPK to this narrow timeframe implies promotion of post-diapause development rather than inhibition of metabolism and development in diapause-destined embryos. These results are intriguing, especially because the reported amount of active AMPK in diapause embryos is different from the quantity in embryos immediately post-diapause and yet to initiate development [76]. Setting AMPK in motion at this time, along with restricted distribution of the enzyme mostly to ectoderm nuclei, is a sign AMPK influences gene expression during the differentiation of post-diapause *Artemia* embryos, rather than exercising metabolic regulation per se.

To my knowledge there are no other reports of AMPK activity in diapausing organisms although a regulatory role is suggested for the enzyme in killifish [26, 48]. As well, quantities of the AMPK-regulated, serine/threonine kinase S6 K in diapause *Artemia* cysts are lower than those in cysts which have just broken diapause, but the effect of the change is uncertain [77]. S6 K from diapause *Artemia* embryos is non-phosphorylated, potentially contributing to termination of mitosis and cell division because it is inactive. Support for the idea that AMPK regulates *Artemia* S6 K phosphorylation is lacking; indeed, experimental evidence points to the contrary (see above). *Artemia* S6 K is however phosphorylated by 12 h post-diapause, when it may assist in re-initiation of mitosis and cell cycle progression [77].

Molecular chaperons as instruments of diapause maintenance in *A. franciscana*: a model system for the study of profound dormancy and stress tolerance

Of paramount significance during diapause is the tolerance of environmental and physiological stressors such as temperature extremes, desiccation, ultraviolet radiation, salinity, hypoxia/anoxia and reduced metabolism. Resistance to stress is enhanced several ways with the synthesis of protective proteins especially important. Among these cell guardians are the molecular chaperones and co-chaperones composed of several protein families separated by amino acid sequence, molecular mass and mechanism of action. These consist of Hsp110 [78], Hsp104 [79], Hsp90 [80–82], Hsp70 [78, 83–85], Hsp60 [85, 86] and the sHSPs [78, 87–89]. The molecular chaperones mediate the folding and compartmentalization of nascent proteins, maintain proteins in functional conformations, protect proteins from irreversible denaturation during stress and aid in their refolding, and assist in the destruction of non-salvageable denatured proteins (Table 1).

Artemia contain several small molecular chaperones (Table 2) and the first to be investigated in detail was p26, initially observed in post-diapause encysted embryos [90, 91]. The p26 protein was subsequently purified to apparent homogeneity and sequenced [92], and its cDNA was cloned and sequenced, revealing a sHSP with a typical α-crystallin domain [93–95]. The *Artemia* p26 gene has been characterized, as have p26 cDNAs from several *Artemia* species of diverse geographical locations and habitats, and all are similar in derived amino acid sequence [96]. p26 forms oligomers, either when synthesized in *Artemia* [90] or in transformed bacteria and transfected mammalian cells [30, 92, 94, 95, 97, 98]. p26 functions as a molecular chaperone in vitro, protecting citrate synthase and *Artemia* tubulin from heat-induced denaturation, and insulin from reduction-induced aggregation [92, 94, 95, 98, 99], while conferring thermotolerance on first instar nauplii hatching from cysts and transformed bacteria [94, 95, 97, 100]. p26 bestows heat resistance on transfected mammalian cells and limits apoptosis induction by heat, drying/rehydration and staurosporine, by impeding procaspase-3 activation [30].

p26 is synthesized only in diapause-destined embryos, appearing at day 3 post-fertilization [100, 101], with p26 mRNA visible a day earlier as determined by probing of Northern blots [100] and quantitative real-time PCR [96]. A very low level of p26 mRNA is observed by PCR in *Artemia* embryos developing directly into nauplii, but p26 protein is not detected by immuno-probing of Western blots [96]. p26 moves into nuclei upon synthesis in diapause-destined embryos, suggesting the chaperone protects both cytoplasmic and nuclear proteins [93, 100–103]. p26 protein and mRNA disappear during post-diapause development, indicating a diapause-specific function for the protein which is last observed in a sub-group of salt gland nuclei in late instar 2 nauplii [90, 100].

As revealed by suppressive subtractive hybridization two other sHSPs, ArHsp21 and ArHsp22 [11, 104, 105], are synthesized in diapause-destined but not nauplius-destined *Artemia* embryos. ArHsp21 and ArHsp22 each contain an α-crystallin domain with a highly conserved arginine, a WXDPF amino-terminal motif duplicated in ArHsp22 and a carboxy-terminal I/VXI/V sequence.

Table 1 Primary functions of molecular chaperones

Chaperone family[a]	Functions	References
Hsp110	Suppress irreversible protein denaturation; nucleotide exchange factor for HSP70; low ATPase activity	[78]
Clp/Hsp100 (Hsp104)[b]	Rescue polypeptides from protein aggregates; facilitate protein refolding or destruction; utilizes ATP	[79]
Hsp90[c]	Promote protein folding, stability, activity and assembly; modulate transcription factors and kinases among other regulatory proteins; influence gene expression and cell signaling; low ATP activity	[80–82]
Hsc70/Hsp70 (DnaK)	Folding of nascent and compromised proteins; protein degradation; clathrin uncoating; protein compartmentalization; stress protection; utilizes ATP	[78, 83–85]
Hsp60 (GroEL, TRiC/CCT, (chaperonins)	Folding of nascent proteins, especially those with multiple domains and/or enriched in β-strands; facilitate oligomer assembly; utilizes ATP	[85, 86]
Small heat shock proteins (sHSPs)	Prevent irreversible protein denaturation during stress; protein refolding and degradation; apoptosis inhibition; cytoskeleton stabilization; no ATPase activity	[78, 87–89]

[a] Although presented in the table as separate families the molecular chaperones function as networks, ensuring that cells contain the appropriate protein complement

[b] Clp/Hsp100 proteins have not been found in animal cell cytosol [79]

[c] *S*-nitrosylation at a highly conserved Cys (597 in humans, 577 in yeast) by NO regulates Hsp90 activity and NO synthesis [80, 82]

Table 2 Diapause-specific *Artemia* molecular chaperones

Property	*Artemia* molecular chaperones			
	p26	ArHsp21	ArHsp22	Artemin
Molecular mass (kDa)	20.8	21.1	22.4	26.0
Amino acid residues	192	181	190	230
α-Crystallin domain (residue numbers)	+(61–152)	+(70–162)	+(74–168)	–
Ferritin homolog	–	–	–	+
Oligomerization	+	+	+	+
Chaperone activity	+	+	+	+
Apoptosis inhibition	+	ND	ND	ND
Developmentally regulated	+	+	+	+
Stress regulated	–	–	+(Adults only)	–
Abundance in cysts	High	ND	ND	High

(Adults only), expression induced by heat only in adults

+ Property observed; – property not observed; *ND* not determined

Quantitative real-time PCR exposed ArHsp21 mRNA in diapause-destined embryos at day 2 post-fertilization followed on day 3 by recognition of its protein product on Western blots, as shown for p26. The appearance of ArHsp22 mRNA and protein was delayed slightly relative to ArHsp21, and neither protein was observed on Western blots containing protein extracts from cyst-derived instar II larvae. ArHsp21 and ArHsp22 oligomerize, whether synthesized in cysts or in transformed bacteria, and they function as molecular chaperones in vitro, preventing heat-induced denaturation of citrate synthase and reduction-induced aggregation of insulin. Of the three *Artemia* sHSPs, only ArHsp21 fails to migrate into nuclei. ArHsp22 synthesis is induced by thermal stress in adults, but not earlier life history stages, whereas neither p26 nor ArHsp21 responds to heat.

The manufacture of p26, ArHsp21 and ArHsp22 in diapause-destined *Artemia* embryos, but not in embryos that develop directly into nauplii, indicates their importance to diapause. All three molecular chaperones potentially protect proteins from irreversible denaturation due to energy limitation and environmental stressors. Functional redundancy provides insurance for diapausing embryos, and at the same time each sHSP may perform task(s) not shared by the others. For example, in contrast to ArHsp22 and p26, ArHsp21 appears not to translocate into nuclei, suggesting that only two *Artemia* sHSPs protect proteins in this organelle. ArHsp22 is induced by heat, making it the only known crustacean sHSP whose synthesis is regulated both developmentally and in response to stress. The latter characteristic suggests an activity for ArHsp22, not shared by p26 and ArHsp21, when *Artemia* encounter environmental stress. p26 inhibits apoptosis, a function possibly limited to this sHSP, although the effects of ArHsp21 and ArHsp22 on apoptosis have yet to be determined. *Artemia* embryos synthesize several additional molecular chaperones such as Hsp70, Hsp90 and Hsp110 [39, 49, 103, 106–110], but their roles in diapause are uncertain. By comparison, dehydration-tolerant diapause embryos of the killifish *A. limnaeus*, which enter a profound dormancy and are resistant to heat, salinity, and anoxia [44], possess a constitutively synthesized Hsp70 with the potential to enhance stress tolerance. Similar possibilities exist for insect molecular chaperones, although insects display diapause characteristics less extreme than those exhibited by *Artemia* and *Austrofundulus*.

Molecular chaperones and insect diapause: diverse strategies with related outcomes

Insects enter diapause as embryos, larvae, pupae and adults, with diapause generally restricted to a single developmental period and characterized by varying degrees of dormancy for each species. Diapause in insects entails differential expression of genes, often demonstrated experimentally by mRNA quantification, and this work has cast light on the role of molecular chaperones as protectors of macromolecules and contributors to stress tolerance. To identify trends in the experimental data, the diapause-dependent expression of genes encoding molecular chaperones is considered at progressively more advanced stages of insect development from larvae to adults (Table 3).

The accumulation of two sHSP mRNAs varies during larval diapause in the corn stalk borer *Sesamia nonagrioides* (Lef.), an organism that feeds and moults throughout the period of reduced activity associated with diapause. *SnoHsp19.5* mRNA remains constant for the duration of diapause, whereas *SnoHsp20.8* mRNA falls off in mid-diapause and expands upon termination, suggesting

Table 3 Molecular chaperones during insect diapause

Development stage	Insect	Molecular chaperone	Regulation	References
Larva	*S. nonagrioides* (corn stalk borer)	sHSP	Constant	[111]
		sHSP	Down	[111]
		Hsc70	Up	[112]
		Hsp70	Down	[112]
		Hsp90	Up	[113]
	O. fuscidentalis (bamboo borer)	Hsc70	Variable	[114]
		Hsp70	Down	[114]
		Hsp90	Down	[114]
	C. suppressalia (rice stem borer)	Hsc70	Constant	[115]
		Hsp90	Up	[115]
	L. sericata (blow fly)	sHSP	Constant	[116]
		Hsp70	Constant	[116]
		Hsp90	Constant/up	[116]
	N. vitripennis (parasitic solitary wasp)	Hsp90	Down	[12]
Pupa	*S. crassipalpis* (flesh fly)	sHSP	Up	[58, 117, 118]
		sHSP	Up	[60]
		sHSP	Up	[60]
		sHSP	Up	[60]
		Hsp60	Up	[60]
		Hsp70	Up	[60, 118, 119]
		Hsp 70	Up	[60, 118, 119]
		Hsc70	Constant	[60]
		Hsp90	Down	[54, 118, 119]
	M. rotundata (solitary bee)	Hsc70	Constant	[120]
		Hsp70	Up	[120]
		Hsp90	Constant	[120]
	D. antiqua (onion maggot)	Hsp60	Up (summer)	[125]
		Hsp60	Up (winter)	[125]
		Hsp70	Up	[121]
		Hsp90	Up (summer)	[124]
		Hsp90	Variable (winter)	[124]
	H. zea (corn earworm)	Hsc70	Constant	[122]
		Hsp70	Constant	[122]
		Hsp90	Down	[122]
	H. armigera (cotton bollworm)	Hsp90	Down	[123]
Adult	*C. pipiens* (mosquito)	Hsc70	Down	[129]
		Hsp70	Constant	[128]
	D. triauraria (fruit fly)	sHSP	Constant	[131]
		sHSP	Constant	[131]
		Hsp70	Constant	[130]
		Hsp90	Constant	[131]
	L. decemlineata (Colorado potato beetle)	Hsp70	Up	[132]
		Hsp70	Constant	[132]

Up increase in HSP during diapause; *down* decrease in HSP during diapause; *variable* HSP increased and decreased during diapause; *constant* no change in HSP during diapause; *constant/up* HSP constant for most of diapause and then increased

mRNA and not protein was generally measured

Summer and winter, diapause during the summer and winter seasons, respectively

different functions for these sHSPs [111]. The amount of *SnoHsp70* mRNA dips as *S. nonagrioides* enters diapause, whereas the cognate *SnoHsc70* mRNA multiplies in deep diapause [112], as does mRNA encoded by the *Hsp83* gene, a homologue of Hsp90 [113]. These observations suggest diapause-related roles for Hsc70 and Hsp90, perhaps in maintaining protein structure and contributing to ecdysone action. The tropical bamboo borer *Omphisa fuscidentalis* undergoes drought-induced larval diapause within the relatively constant environment of the bamboo culm internode [114]. *Hsp70* mRNA drops in *O. fuscidentalis* fat bodies as diapause initiates and remains depressed into the pupal stage, while *Hsc70* mRNA is low for the first 4 months of diapause before experiencing a 12-fold amplification prior to decreasing. The quantity of *Hsp90*, a molecular chaperone that regulates cell signaling through interaction with metastable proteins [82], is unchanged for much of *O. fuscidentalis* diapause after going through pre-diapause fluxes, and then it declines before exhibiting transient growth at termination [114]. The authors conclude that *O. fuscidentalis Hsp70* is not associated with diapause although *Hsp90* and *Hsc70*, the latter possibly under 20-hydroxyecdysone and juvenile hormone control, are. During larval diapause of the rice stem borer, *Chilo suppressalia*, in the same family as *O. fuscidentalis*, *Hsp90* but not *Hsc70* mRNA builds up [115]. mRNAs encoding *Hsp23*, *Hsp70* and *Hsp90* are unchanged in the non-feeding, third instar larval diapause of the blow fly, *Lucilia sericata*, however *Hsp90* transcripts proliferate as diapause terminates, perhaps in response to ecdysteroid [116]. The changes in *Hsp90* may reflect increased signaling activity as cells return to pre-diapause activity [82, 116]. In comparison, *N. vitripennis* Hsp90 protein decays as larval diapause progresses [12].

Diapause is induced in pupae of the flesh fly *S. crassipalpis* via failure of the brain to promote ecdysteroid production [58]. As made evident by the probing of Northern blots containing RNA from either the entire organism or the brain, transcripts encoding a 23 kDa sHSP (*Hsp23*) increase significantly upon initiation of flesh fly diapause, remain high throughout this life phase and then decrease as diapause ends [58, 117, 118]. At least three other sHSPs up-regulate during *S. crassipalpis* diapause [60]. Two different transcripts encoding inducible *Hsp70* also rise as flesh flies enter diapause, declining upon termination [60, 118, 119]. Similar results pertain for *Hsp70* during the pre-pupal diapause of the solitary bee *Megachile rotundata* [120] and the summer and winter pupal diapauses of the onion maggot *Delia antiqua* [121]. *Hsc70* mRNA accumulation is however unchanged when *S. crassipalpis* and *M. rotundata* pass through diapause and in the corn earworm, *Helicoverpa zea*, neither *Hsp70* nor *Hsc70* mRNA is affected by diapause [122].

Hsp90 mRNA ebbs throughout *S. crassipalpis* diapause, returning to higher levels when development resumes [54, 118, 119]. The drop in *S. crassipalpis Hsp90* mRNA may be attributed to ecdysone reduction, which leads to the coordinated increase of *Hsp23* and *Hsp70* mRNA, suggesting the same regulatory mechanisms for these genes. The amount of Hsp90 protein declines in pupal heads from the diapausing cotton bollworm, *H. armigera* [123], and *Hsp90* mRNA is down-regulated in *H. zea* [122]. In contrast *M. rotundata Hsp90* mRNA is constant [120], and *D. antiqua Hsp90* mRNA accumulates gradually during summer diapause, fluctuates throughout winter diapause after an initial pre-diapause boost and regresses at termination [124]. The quantity of *D. antiqua* mRNA encoding t-complex polypeptide-1 (TCP-1), a subunit of the chaperonin CCT or *Hsp60*, grows during summer diapause and declines as development resumes, whereas in winter diapause the quantity of mRNA for this protein expands [125]. It was proposed, based on work with other organisms, that *Hsp60* contributes to the robust cold hardiness of *D. antiqua* by interaction with the cytoskeletal elements actin and tubulin [124]. Perhaps *Hsp60* has a similar function in *S. crassipalpis* [60], as well as in *A. franciscana* as a component of the microtubule proteome [126], and in *Daphnia magna* where Hsp60 is greater, relative to total protein, in diapause versus subitaneous eggs [127].

Hsp70 mRNA is not augmented in diapausing adults of the mosquito *C. pipiens*, even though they are more cold and desiccation tolerant than fully active adults [128], and the amount of Hsc70 protein declines in the heads of adult *C. pipiens* females in early diapause [129]. Nor does *Hsp70* mRNA increase in *Drosophila triauraria*, even though the organism acquires enhanced stress tolerance upon entering reproductive or adult diapause [130]. Moreover, as revealed by RT-PCR, mRNA encoded by *D. triauraria Hsp23*, *Hsp26* and *Hsp83*, the latter equivalent to *Hsp90*, do not experience diapause-associated transcriptional regulation [131]. The Colorado potato beetle, *Leptinotarsa decemlineata*, exhibits differential expression of inducible *Hsp70* transcripts, with *LdHSP70A* expression low during adult diapause and *LdHSP70B* mRNA not detectable [132].

To summarize, not all classes of molecular chaperones contribute to diapause in each insect species, nor does every type of chaperone function at each diapause stage. Universal participation by chaperones is not required, however, to preserve the notion of their importance during diapause, even in insects which remain relatively active in this phase of their life cycle and have reduced need for protein protection. There are, of course, caveats to this proposal. It is difficult to comment definitively upon the relevance of a particular group of molecular chaperones during diapause until all members of the family are examined, especially if only RNA is analyzed. In spite of

this, analysis of mRNA accumulation indicates that diapause induction, maintenance and termination may differently affect the expression of molecular chaperone gene family members. Additionally, the expression of different genes evokes functional heterogeneity among individual stress proteins within and between families, although there is sharing of activities as well. For example, in a general sense, many molecular chaperones protect diapausing insects against environmental and physiological stressors, as shown for flesh fly *Hsp70* and *Hsp23*, but their mechanisms of action and the proteins/processes affected may vary. The sHSPs prevent irreversible aggregation of partially denatured proteins, holding them for renaturation or destruction by other chaperones [78, 88, 89]. Members of the Hsp70/Hsc70 family assist compromised or nascent proteins to attain their native three-dimensional conformation and may work with the sHSPs to this end [85, 89], whereas Hsp60, operating in concert with other chaperones, is likely to mediate the folding of specific proteins such as actin and tubulin [85, 86]. Hsp90, on the other hand, an ATP-requiring dimeric chaperone seldom observed to increase in diapause insects, contributes to cell signaling and gene expression rather than exhibiting a blatant protective role through interaction with denaturing proteins [80, 81]. Decreasing Hsp90 mRNA during diapause reflects reduced need for a chaperone that stabilizes proteins involved in cellular signaling because metabolism is decreasing and dormancy ensues. The molecular chaperones appearing during insect diapause therefore tend to reflect organismal needs, this being a manifestation of changing physiological needs as diapause progresses.

Artemin, a diapause-specific ferritin homologue with chaperone activity

In addition to the more readily recognized HSPs, *Artemia* cysts contain artemin (Table 2), an abundant protein first shown to resemble ferritin upon sequencing by Edman degradation, but with a high content of cysteine and histidine [133]. cDNA cloning and sequencing confirmed the similarity between artemin and ferritin [134]. Artemin, a heat-tolerant protein, has a monomeric molecular mass of approximately 26 kDa, and it forms oligomers of approximately 600 kDa or 24 subunits [133, 135, 136]. The synthesis of artemin is developmentally regulated, appearing by day 4 post-fertilization in the cytoplasm of diapause-destined *Artemia* embryos. Artemin disappears during post-diapause growth and is not found in embryos developing directly into nauplii, signifying a role in cyst development and diapause [107, 108].

Unlike ferritin, artemin fails to sequester iron even though it fashions oligomers of equivalent molecular mass [134, 136]. Artemin has a carboxy-terminal amino acid extension not found in ferritin, which is thought to fold inward, filling the space within oligomers that would normally be available to house iron. Five of the six di-iron ferroxidase center residues required for iron storage are also missing from artemin. If artemin does not bind iron, what is its function? Upon heating artemin interacts with RNA, suggesting the protein is an RNA chaperone, a potentially important function during diapause [135]. Artemin purified from transformed bacteria protects citrate synthase from heat-induced denaturation in a concentration-dependent manner, a characteristic surprisingly shared by ferritin and apoferritin, while transfected mammalian cells expressing artemin exhibit enhanced tolerance to heating and oxidative stress [136]. These results, in concert with those from in vitro turbidimetric assays, demonstrate that artemin and ferritin, like p26 and other *Artemia* sHSPs, are molecular chaperones. As a working hypothesis, artemin arose from ferritin by gene duplication, gradually losing the ability to store iron, but retaining chaperone activity. Accumulation to high levels implies artemin has an important role in diapause-destined *Artemia* embryos as a molecular chaperone, a sustainable activity because artemin no longer binds metals and would not sequester intracellular iron required by active post-diapause embryos.

Artemin may be unique to *Artemia* and its close relatives, but ferritin up-regulates in other diapause-destined organisms such as the copepod, *Calanus finmarchicus* [38], the wasp, *N. vitripennis* [12] and the rotifer, *B. plicatilis* [14]. There are many interesting questions regarding diapause-related ferritin functions. Does ferritin possess molecular chaperone activity in vivo, and if so, does it interact with a restricted number of molecular clients because it is present in small amounts even after up-regulation? On another tack, by increasing ferritin the amount of free iron is reduced leading to suppression of cell growth and division, important diapause characteristics. Ferritin scavenges oxygen radicals and by sequestering iron may curb the Fenton reaction, preventing ROS build-up. As a consequence, cellular macromolecules are protected from oxidative damage and intracellular signaling is influenced [12, 14, 34], as considered in the next section of the review.

Reactive oxygen and nitrogen species: from macromolecule perturbation to cell regulation

Brachionus plicatilis undergoes profound dormancy, and analysis of EST libraries prepared from this organism indicates an abundance of mRNAs encoding anti-oxidant proteins such as glutathione *S* transferases, dismutases, peroxiredoxins and thioredoxin peroxidase [14]. Such observations suggest the importance of controlling intracellular redox potential during diapause, but work in this area is limited. Free radicals, highly reactive chemical

species possessing an unpaired electron(s), are generated by the loss or gain of free electrons by oxygen, resulting in production of ROS (Fig. 2a) [34] and RNS (Fig. 2b) [137]. Within the family of common biologically significant ROS the superoxide radical is relatively unstable, and it may form H_2O_2, a non-radical ROS, which if not reduced to H_2O by catalase gives rise to the hydroxyl radical via the Fenton reaction [34]. ROSs have long been recognized as purveyors of oxidative damage, with the hydroxyl radical the most destructive. ROS-induced insults include lipid peroxidation, mutations, DNA strand breakage and post-translational modification/inactivation of proteins, changes with the potential to cause cell perturbation and death [138]. The major enzymatic mechanisms that protect cells against excessive ROS, some of which also have a role in signaling (see below), entail interrelated actions by glutathione *S* transferase, superoxide dismutase, catalase and peroxidases [14, 139, 140], among other effectors. Hsp70 and sHSPs are non-enzymatic modulators of ROS damage, and their synthesis is regulated transcriptionally by heat-induced H_2O_2 production, suggesting cross-talk between ROS and molecular chaperones [141, 142]. Genes encoding protective proteins may be differentially up-regulated during dormancy and growth in unfavorable conditions [14, 138, 143, 144]. Another major regulator of intracellular ROS accumulation is the abundant tripeptide glutathione (GSH) (L-γ-glutamyl-L-cysteinyl–glycine), a reducing agent with the ability to prevent apoptosis [145]. sHSPs increase reduced glutathione by up-regulating enzymes such as glucose-6-phosphate dehydrogenase, instrumental in the regeneration of NADPH from $NADP^+$, the former conscripted by GSH reductase to reduce glutathione-disulfide (GSSG) to GSH [143–145]. Dangers attributed to ROS increase during seed dehydration and dormancy [22], suggesting protection against oxidative damage is valuable to organisms that experience profound dormancy and dehydration/desiccation as part of diapause, a proposal awaiting further investigation.

In addition to destructive potential, ROSs function as signaling molecules required for cell growth, division and development. The NADPH oxidases, a family of stress-activated integral membrane proteins, generate H_2O_2 thought to act as a second messenger [19, 139, 146]. Regulatory levels of H_2O_2 and superoxide anion are manufactured during respiratory activity, photosynthesis and lipid peroxidation in dry tissues such as seeds [22]. To effect signaling H_2O_2 oxidizes thiol-containing protein residues, perhaps via protein oxidation relays where peroxidase as primary oxidant receptor transfers oxidation to target proteins [147], ultimately promoting tyrosine phosphorylation and dephosphorylation. Protein tyrosine phosphatases, active in cell metabolism, growth and differentiation are targeted by H_2O_2, as are mitogen-activated protein kinases (MAPKs) and transcription factors, thereby controlling molecular processes ranging from gene expression to protein posttranslational modification [19, 20, 146, 148]. The consequences of possessing, such a wide range of influences at the molecular level, are many, and as one example H_2O_2 alleviates seed dormancy and promotes germination by transmitting environmental information within cells [22, 139]. Exposure to H_2O_2 terminates *Artemia* cyst diapause [149], but molecular investigations of this process are lacking. In the same context, *B. plicatilis* requires light for post-diapause development and hatching [150]. Light may promote development by producing ROS in sea water an idea corroborated by H_2O_2-induced development of *B. plicatilis* in darkness, perhaps as the result of prostaglandin synthesis due to lipid oxidation [150]. On a different but still beneficial note as far as diapause is concerned, ROS, including H_2O_2, inhibit caspases and other apoptosis effectors [146]. This is a decided advantage because slowing of cell death, especially upon stress exposure, prolongs the well-being of dormant organisms.

The RNS nitric oxide (NO) (Fig. 2b) is produced in the mitochondrial respiratory chain [137], through the action of NO synthase, which converts L-arginine to L-citrulline [151], via nitrate reductase [152] and as a result of enzymatically mediated nitrite reduction [153, 154]. NO reacts with superoxide to generate peroxynitrite, a potent oxidizer of nucleic acids and proteins [137, 155, 156]. RNS are responsible for S-nitrosylation (*S*-nitrosation), a reversible protein modification, as well as nitrotyrosine production, a potentially non-reversible change subject to H_2O_2 modulation [19, 151, 157, 158]. The post-translational *S*-nitrosylation of proteins entails the covalent interaction of NO with a cysteine thiol group [156, 157, 159, 160], and if excessive due to NO over-production *S*-nitrosylation causes protein mis-folding. The *S*-nitrosoglutathione reductase and thioredoxin systems are denitrosylases that protect against too much protein *S*-nitrosylation, a situation known as nitrosative stress [160].

In a regulatory capacity, NO promotes cyclic 3′,5′-guanosine monophosphate (cGMP) production, a second messenger that impacts ion channel activity, membrane receptors and signaling cascades through the actions of kinases and phosphatases [151, 157]. Posttranslational *S*-nitrosylation and its reversal or denitrosylation driven by the *S*-nitrosoglutathione reductase and thioredoxin systems, also affect membrane transporters and receptors, transcription factors, multiple enzymes and structural proteins. As a consequence, physiological processes are regulated in a redox-dependent manner, but with protein specificity as opposed to the global influences exerted by the cell redox state [156, 157, 159, 160]. Protein modification by NO modulates several cellular and organismal processes

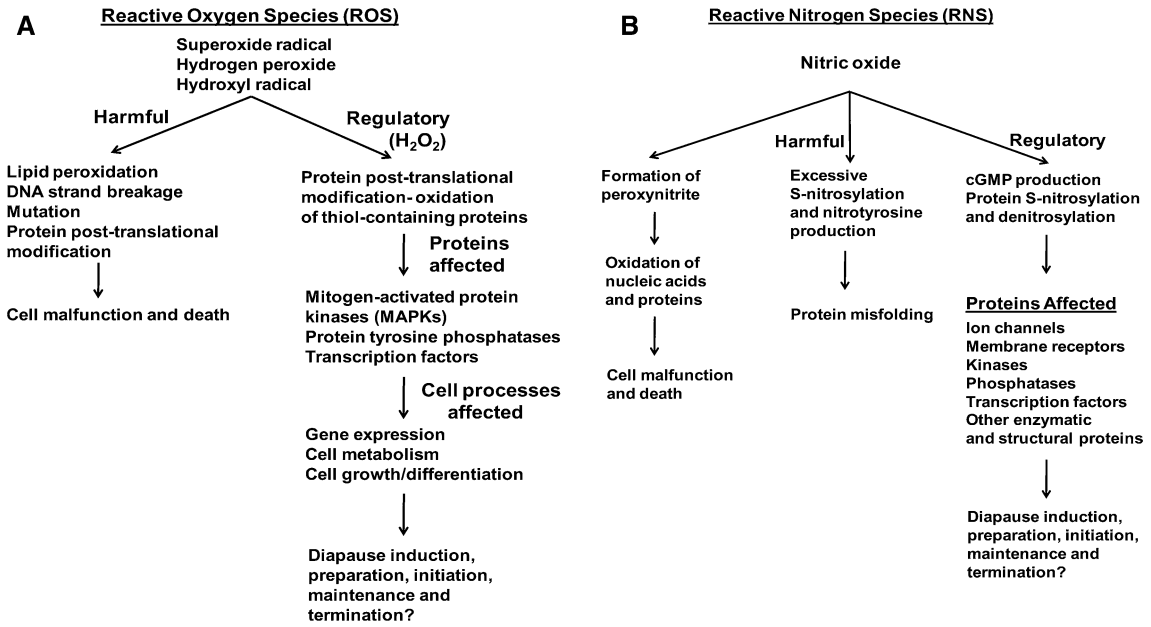

Fig. 2 Regulation of diapause by ROS and RNS. When produced in excess, ROS and RNS damage macromolecules leading to cell malfunction and death. In contrast, both ROS (H_2O_2) and RNS (NO) act as, or modulate, second messengers resulting in posttranslational modifications which affect protein activity and cell behavior, changes with the potential to influence diapause, although this has yet to be thoroughly investigated at the molecular level

including the obstruction of apoptosis by caspase *S*-nitrosylation at the active site Cys [155, 157], this of value to animals undergoing diapause. Moreover, in plant seeds, NO enhances release from dormancy through transcriptional regulation of the gene *CYP707A2*, which encodes (+)-abscisic acid 8′-hydrolase, an enzyme that degrades abscisic acid (ABA) [161]. Although this is very unlikely to be the mechanism responsible for breakage of diapause-associated dormancy in animals, potential clearly exists for NO and other RNS to control diapause and development through second messenger production, gene regulation and protein posttranslational modification.

Apoptosis during diapause

Apoptosis is a genetically regulated process of programmed cell death induced by energy limitation among other stressors, and characterized by activation of cysteine proteases, or caspases. If organisms are to live through energy deprivation upon exposure to environmental hardship or entry into diapause, apoptosis must be curtailed. In this way cell integrity is maintained, permitting development when diapause terminates and embryos return to favorable growth conditions. In *A. franciscana* embryos, for example, diapause gives rise to profound reduction in metabolism, arguably to a reversible standstill [47, 162]. During this time, and upon exposure of post-diapause encysted embryos to prolonged anoxia, internal ultrastructure and macromolecules are preserved, indicating inhibition of proteolytic activity and apoptosis [90, 107, 162–164]. These findings are remarkable because energy limitation can stimulate liberation of pro-apoptotic factor(s) via opening of mitochondrial permeability transition pores, activating caspases and causing cell death [165]. However, the Ca^{++}-induced opening of mitochondrial pores commonly observed during apoptosis is lacking in *Artemia* although their mitochondria contain the main pore structural proteins, and cytochrome *c* is not released during diapause and anoxia [31, 165–167]. In addition, neither *Artemia* caspase-9 nor caspase-3 is regulated by cytochrome *c*, as occurs in mammalian cells, but caspase-9 activity is increased by exogenous Ca^{++} and decreased by the nucleotides ATP, GTP and ADP, with the latter providing the greatest inhibition [31, 167]. ADP appears not to decline in diapausing *Artemia* embryos, and this nucleotide may come into play when other apoptosis inhibitory nucleotides are reduced [31].

Apoptosis is controlled by sHSPs the ubiquitously distributed molecular chaperones described earlier. sHSPs inhibit programmed cell death by interacting with β-arrestin [168], p53 [169], pro-apoptotic members of the Bcl-2 family such as Bax and Bcl-Xs [170], cytochrome *c* [143] and caspases [171, 172]. Notably, the diapause-specific sHSP p26 synthesized by *Artemia* embryos curtails apoptosis in stably transfected mammalian cells upon exposure to heat, staurosporine and dehydration/rehydration [30]. Inhibition may occur via p26 interaction with pro-apoptotic proteins and/or caspases that have yet to be characterized in

Artemia, although binding with cytochrome *c* appears not to be involved [167]. Apoptosis management potentially benefits cells in energy limited states and represents one way in which *Artemia*, and perhaps other organisms, survive the physiological stressors, anoxia and diapause.

Water stress tolerance during diapause

Although not an overly common characteristic, some bacteria, plants and animals successfully endure drying or desiccation as part of their normal life cycle, a property often dependent on small molecules such as trehalose and sucrose. These disaccharides are thought to replace water that is hydrogen-bonded to polar residues, enabling proteins and membranes to retain physical properties similar to those of the fully hydrated state and promoting survival during water stress [173–175]. For example, trehalose hydroxyl groups hydrogen bond with membrane phospholipid head groups inhibiting rehydration-induced lipid phase transitions [36, 176]. Sugars also produce a high viscosity cytoplasmic glassy matrix, reducing molecular mobility and protecting embedded organelles [174, 177, 178]. Diapause-destined embryos are the only *Artemia* life stage to accumulate trehalose, this commencing 2 days post-fertilization and ending with metabolic cessation after cyst release from females [179, 180]. Trehalose may protect *Artemia* embryos against water stress, serve as an energy reserve for post-diapause development, and as a substrate to generate glycogen and glycerol [46, 177]. The nematode, *Aphelenchus avenae*, abundantly accumulates trehalose, contributing to its endurance during desiccation-induced dormancy or anhydrobiosis [178]. Larvae of the Antarctic midge, *Belgica antarctica*, possess several adaptations facilitating survival upon frequent exposure to conditions that cause water loss and one of these is trehalose generation [181]. Trehalose build up is thought to be essential for continued existence of *B. antarctica*, but this mechanism is not employed by all organisms that tolerate dehydration or cold. For example, resting eggs of the rotifer *B. plicatilis* are not enriched in mRNA encoding trehalose-6-phosphate, a key enzyme in trehalose synthesis, leaving the role of this disaccharide uncertain even though the organism lives through desiccation [14], nor does trehalose increase in overwintering diapause larvae of the rice stem borer *C. suppressalis* [182].

Glycerol, possibly arising from glucose and glycogen, functions as a cryoprotectant by reducing freezing temperature, curbing ice crystal growth and shielding against cold-induced macromolecule disruption [3, 127, 181, 182]. Glycerol potentially has a protective role in diapausing organisms. As a case in point, glycerol is more plentiful in dormant as opposed to subitaneous eggs of *D. magna* where it either protects against stress or represents a substrate suitable for metabolism when growth resumes [127]. Glycerol quantities expand in diapausing larvae and pupae, respectively, of *C. suppressalis* [115], and *S. crassipalpis*, reaching a concentration of 110 mM. This level is maintained in diapause pupae although it declines upon diapause termination and the initiation of quiescence [118]. Glycerol also peaks in diapause larvae of *C. suppressalis,* with maximal accumulation coinciding with the most intense diapause and greatest cold hardiness [182].

In addition to small molecules, the LEA proteins, first observed in developing cotton seeds [183], augment protection against desiccation and cold [34, 35]. The LEA proteins occur in organisms other than plants including the bacterium, *Bacillus subtilis* [184], bdelloid rotifers capable of anhydrobiosis [185], and at least two other invertebrates which experience very different diapauses, namely *C. elegans* [186] and *A. franciscana* (Fig. 3) [36, 37, 187]. Nematodes successfully withstand desiccation, and exposure of *C. elegans* dauer larvae to dehydration amplifies accumulation of the LEA protein group 3 mRNA encoded by the gene *Ce-lea-1*. Partial silencing of *Ce-lea-1* diminishes survival during desiccation, showing LEA proteins contribute to drying tolerance [186]. Protection by LEA proteins during water stress is additionally suggested by the amplification of RNAs encoding two highly hydrophilic group 3 LEA proteins in diapause-destined *Artemia* embryos [188, 189]. The mRNA for a third group 3 LEA protein termed AfrLEA3 is enriched in desiccation-tolerant diapause *Artemia* embryos. AfrLEA3 is thought to protect mitochondria against water loss, with naturally occurring intra-mitochondrial trehalose having a supplementary role [36, 187]. One 21 kDa, heat stable, group 1 LEA proteins reside in encysted *Artemia* embryos but not in larvae and adults, the first time these proteins have been observed in animals [37]. The *Artemia* group 1 LEA proteins protect citrate synthase against drying in vitro, an action boosted significantly by trehalose, but not against heat. Enhancement of LEA protein effects by trehalose was reported previously and may involve stabilization of the sugar-based glassy matrix characteristic of desiccation-tolerant organisms [33–35, 174, 178].

Late embryogenesis abundant proteins guard against salt and cold stress, chilling and freezing, and water stress, with activities differing from organism to organism and for one LEA protein versus another [35]. How LEA proteins fulfill diverse roles is uncertain, but functional promiscuity undoubtedly derives in part from their hydrophilicity and limited secondary structure [35, 37, 190]. Stress tolerance is thought to arise in different ways, not all shared by each LEA protein, and various possibilities are summarized in two excellent reviews [34, 35]. LEA proteins could act as "molecular shields," which decrease interaction between and aggregation of denaturing proteins, and they may

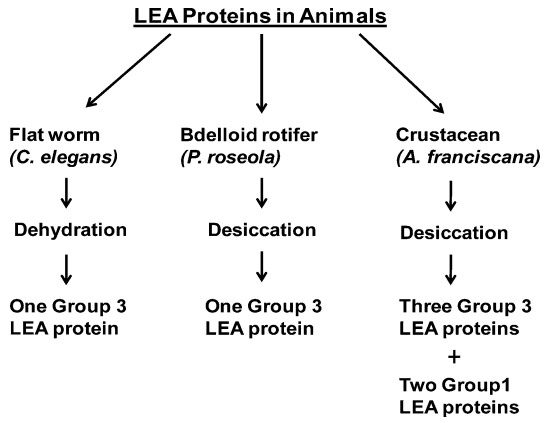

Fig. 3 LEA proteins in animals. LEA proteins, first observed in plants, have been reported in three animals where they may provide tolerance against water stress. Group 3 LEA proteins are found in all three animals, whereas only *A. franciscana* exhibits group 1 LEA proteins

generate space filling filaments thereby enabling cells to avert collapse as water content decreases. The hydrophilic character of LEA proteins allows them to sequester ions, preventing oxidative stress when ROS and the metals that influence their production are scavenged. Lastly, LEA proteins have the potential to interact with and fortify membranes, including those of mitochondria, where they reduce oxidative phosphorylation.

Molecular chaperones such as the *Artemia* sHSP p26 [23] and artemin [136] have the potential to protect against protein damage due to water loss from cells, but this has yet to be tested in embryos. In support of this proposal, however, p26, acting synergistically with trehalose, defends against drying-induced damage in transfected mammalian cells [191], suggesting a similar role for sHSPs in *Artemia* and other animals where these chaperones accumulate.

Protective coverings—morphological adaptations associated with diapause

Protective coverings, synthesized by organisms undergoing various types of dormancies [9, 28, 40, 41, 150], represent major diapause-related morphological adaptations, but their study is mostly descriptive. *A. franciscana* cysts are surrounded by a thick, non-cellular, chitinous shell that confers tolerance to ultraviolet radiation, a stress experienced when cysts float on the water or are blown on shore and exposed to sun light [108, 161]. *Artemia* cyst shell ultrastructure is well described, and this structure has similarities to insect egg shells [189, 192–194]. The multi-layered cyst shell shows species-dependent structural variability, but generally there is an external, darkly pigmented chorion layer consisting of maternally derived cortical and alveolar layers susceptible to hypochlorite degradation. The chorion is separated from the hypochlorite-resistant embryonic cuticle, composed of a fibrous layer containing protein and chitin, and the inner cuticular membrane of embryonic origin by a thin membrane termed the outer cuticular membrane. The intact cyst shell is impermeable to non-volatile substances, a property conferred in part by the outer cuticular membrane, a structure essential to embryo development [192, 195]. Removal of the outermost chorion, a process called dechorionation, opens the way for passage of lipid-soluble molecules into embryos. This observation demonstrates the effect of the chorion on cyst permeability and, importantly, that limited pharmacological approaches can be used to analyze embryo activities [196, 197].

Up-regulated mRNAs in ovisacs of *A. parthenogenetica* containing embryos destined for diapause have been selected by suppressive subtractive hybridization as a way to examine cyst shell synthesis and function. One of the cDNAs obtained represents a gene (*SGEG*) expressed specifically in the shell gland and it encodes a pro-protein with a signal sequence of 33 amino acids and a 14 kDa mature protein of 81 residues. Knockdown of the *SGEG* product by RNAi yields transparent cysts with soft yellow shells containing mal-formed cortical and alveolar layers [29]. Approximately 20% of the abnormal cysts hatched without termination of diapause by drying or cold. Moreover, *Artemia* cysts with reduced *SGEG* product are less resistant to drying, osmotic pressure, UV radiation, heat and organic solvents, and hatching success upon diapause termination suffers. The *SGRG* product is obviously required for cyst integrity because shell structure is compromised and protective properties decrease when the protein dwindles, providing the first molecular description of cyst shell protein function. Lack of the SGEG protein may also cause p26 and artemin decline, which compromises cyst stress tolerance [29]. These results potentially represent an example of coordinated gene regulation where proteins required for different aspects of diapause maintenance are synthesized in concert, maybe in response to a common transcriptional regulator.

Diapause II embryos of *A. limnaeus*, the most stress-tolerant of three killifish developmental stages to enter diapause, possess the highest known resistance to drying among aquatic vertebrates [28]. Characteristically, water exit from diapause II embryos is high at first and decreases to very low values; the initial water loss, mainly from the perivitelline fluid, almost completely eliminates this space. The enveloping embryo cell layer or chorion has low permeability which assists in limiting water and ion exodus [198]. The biochemical basis of reduced chorion permeability has yet to be determined, but the egg envelope of all *A. limnaeus* diapause life history stages contains long

un-branched protein fibrils similar to amyloid fibers associated with human neurological diseases, including a high content of intermolecular β-sheet. Even though an intact chorion is required it appears this layer is not responsible for the enhanced survival of diapause II embryos. Rather, the perivitelline space is thought to form a hydrophobic barrier or to vitrify, reducing water loss [28] and posing an unusual protective structure very different from the one surrounding encysted *Artemia* embryos. In another example, proteomic analysis of heads from adult females of *C. pipiens* revealed that the pupal cuticle protein increased during early diapause and then decreased [129]. The enhanced amount of pupal cuticle protein may provide a harder cuticle, adding to the stress tolerance of overwintering animals.

Conclusions and perspectives

Diapause is an intriguing developmental process occurring in a myriad of species and characterized by properties so diverse that defining the crucial phenotypic attributes of the process is difficult. Even well-acknowledged diapause properties such as dormancy, including cessation of development and metabolic down turn, enhanced stress tolerance and the need for a termination signal before development resumes vary from extreme to non-existent. Diapause therefore represents a continuum of physiological states potentially generated by the differential application of similar molecular mechanisms in one organism versus another, and with this in mind selected regulatory and physiological parameters with the potential to modulate diapause were considered. The intention of the review was to describe unifying cell/molecular properties of diapause, to the extent they exist, with a good example being the role of molecular chaperones in stress resistance. Organisms exhibiting extreme dormancy and stress tolerance during diapause tend to accumulate large amounts of molecular chaperones, while organisms that change little tend to exhibit much smaller quantities of these proteins, if any at all. Other processes with less established roles in diapause such as AMPK-dependent regulation of metabolism and ROS/RNS signaling were also considered, revealing a need for their in depth examination. Such studies, and especially those driven by the application of sophisticated cell/molecular technologies, will bring to light new properties of diapause. Not only is this important from fundamental perspectives related to cell growth, signaling and stress tolerance, but implications exist for agriculture, forestry, aquaculture and medicine. Clearly, there is much to anticipate as the study of diapause expands and the secrets of this physiological process are uncovered.

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