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Embryonic diapause: development on hold

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ABSTRACT Embryonic diapause, the temporary suspension of development of the embryo, is a fascinating reproductive strategy that has been frequently exploited across the animal kingdom. It is characterized by an arrest in development that occurs at the blastocyst stage in over 130 species of mammals. Its presumed function is to uncouple mating from parturition, to ensure that both occur at the most propitious moment for survival of the species. Diapause can be facultative, i.e. induced by physiological conditions, or obligate, i.e. present in every gestation of a species. In the latter case, the proximal signals for regulation are related to photoperiod. Three diverse models, the mouse, the mustelid carnivores and the wallaby have been studied in detail. From these studies it can be discerned that, although the endocrine cues responsible for induction of diapause and re-initiation of development vary widely between species, there are a number of commonalities. Evidence to date indicates that the uterus exercises the proximal regulatory influence over whether an embryo enters into and when it exits from diapause. Some factors have been identified that appear crucial to this regulation, in particular, the polyamines. Recent studies indicate that diapause can be induced in species where it does not exist in nature. This suggests that the potential for diapause in mammals to be due to a single evolutionary event, to which control mechanisms adapted when the trait was beneficial to reproductive success. Further work at the molecular, cellular and organismic levels will be required before the physiological basis of diapause is resolved.

KEY WORDS: *blastocyst, diapause, polyamine, evolutionary strategy*

Introduction

Reversible arrest of embryo development occurs across the animal kingdom, with multiple examples in nematodes, insects, non-mammalian vertebrates and mammals. The phenomenon, known as embryonic diapause, has intrigued biologists of several stripes, from those focused on evolution, to embryologists, endocrinologists and animal scientists. The first recorded observation of diapause is found in the field notes of William Harvey (1578-1657), who accompanied King Charles I on hunts of roe deer, where he observed differential states of embryonic development following examination of uterine contents (Hunter, 1995). The first report in scientific literature for mammals appears to be that of Bischoff in 1854, on the roe deer (cited in Sempere, 1977), followed by that of Lataste in 1891, who showed lengthened gestation in female rodents suckling large litters (cited in Psychoyos, 1992). In rodents and in many other mammalian species (Table 1), the embryo is arrested at the blastocyst stage for variant intervals that are specific to the species and to the environmental conditions. A similar strategy known as delayed development is found in bats, whereby the blastocyst implants superficially, then grows very slowly, resulting in long and variable gestation lengths (Heideman, 1989). Although there are some detracting hypotheses, the consensus view of the significance of embryonic diapause as an evolutionary strategy is that it allows the uncoupling of fertilization from birth, thereby ensuring that postnatal development occurs under the most favorable environmental conditions for survival of the offspring (Lindenfors *et al*., 2003).

Embryonic diapause has been identified in over 130 mammalian species (Table 1) and the range of external mechanisms and processes acting on the uterine environment during this time can differ significantly amongst species. Despite this, many of the molecular controls of embryonic diapause appear to have been conserved. However, the precise mechanisms by which the uterus exerts its influence on the embryo are still not clearly understood.

Abbreviations used in this paper: CB1, CB2, Cannabinoid receptors; Esr1, Estrogen receptor-α; Esr2, Estrogen receptor-β; FGF, Fibroblast growth factor; HBEGF, Heparin binding epidermal growth factor; IL1B, Interleukin 1β; LIF, Leukemia inhibitory factor; miRNA, MicroRNA; Msx1, Muscle segment homeobox-1; Msx2, Muscle segment homeobox-2; ODC1, Ornithine decarboxylase; Pgr, Progesterone receptor; Wnt, Wingless gene family.

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Furthermore, since it is unknown whether the evolution of diapause was a single event or if it evolved multiple times, it is unclear to what extent the mechanisms by which the embryo-uterine dialogue coordinates the events of diapause are conserved across species.

These cautions notwithstanding, the study of embryonic diapause has the potential to provide us with a greater understanding of many aspects of early embryo development. For example, determining how an embryo is able to remain quiescent for an extended period of time could elucidate the minimum factors required for embryo survival both *in vivo* and *in vitro*. Similarly,

TABLE 1

LIST OF CURRENTLY KNOWN SPECIES WITH PRE-IMPLANTATION EMBRYONIC DIAPAUSE

Order	Family	Common name	Species
OBLIGATE			
EUTHERIA			
Artiodactyla	Cervidae	Roe deer	*Capreolus capreolus*
Carnivora	Mephitidae	Hooded skunk	*Mephitis macroura*
		Striped skunk	*Mephitis mephitis*
		Western spotted skunk	*Spilogale gracilis*
	Mustelidae	Hog-badger	*Arctonyx collaris*
		American hog-nosed skunk	*Conepatus mesoleucus*
		Sea otter	*Enhydra lutris*
		Wolverine	*Gulo gulo*
		North American otter	*Lontra canadensis*
		Neotropical river otter	*Lontra longicaudis*
		American marten	*Martes americana*
		Yellow-throated marten	*Martes flavigula*
		Beech marten	*Martes foina*
		Nilgiri marten	*Martes gwatkinsii*
		European pine marten	*Martes martes*
		Japanese marten	*Martes melampus*
		Fisher	*Martes pennanti*
		Sable	*Martes zibellina*
		European badger	*Meles meles*
		Honey badger	*Mellivora capensis*
		Short tailed weasel (stoat)	*Mustela erminea*
		Long tailed weasel	*Mustela frenata*
		European mink	*Mustela lutreola*
		American mink	*Neovison vison*
		American badger	*Taxidea taxus*
		European marbled polecat	*Vormela peregusna*
	Odobenidae	Walrus	*Odobenus rosmarus*
	Otariidae	New Zealand fur seal	*Arctocephalus forsteri*
		Antarctic fur seal	*Arctocephalus gazella*
		South African fur seal	*Arctocephalus pusillus*
		Subantarctic fur seal	*Arctocephalus tropicalis*
		Northern fur seal	*Callorhinus ursinus*
		Stellar sea lion	*Eumetopias jubatus*
		Australian sea lion	*Neophoca cinerea*
		Southern sea lion	*Otaria byronia*
		California sea lion	*Zalophus californianus*
	Phocidae	Hooded seal	*Cystophora cristata*
		Bearded seal	*Erignathus barbatus*
		Grey seal	*Halichoerus grypus*
		Weddell seal	*Leptonychotes weddellii*
		Crabeater seal	*Lobodon carcinophagus*
		Northern elephant seal	*Mirounga angustirostis*
		Southern elephant seal	*Mirounga leonina*
		Ross seal	*Ommatophoca rossi*
		Harp seal	*Pagophilus groenlandicus*
		Ribbon seal	*Phoca fasciata*
		Ringed seal	*Phoca hispida*
		Spotted seal	*Phoca largha*
		Baikal seal	*Phoca sibirica*
		Harbour seal	*Phoca vitulina*
	Ursidae	Giant panda	*Ailuropoda melanoleuca*
		Spectacled bear	*Tremarctos ornatus*
		Black bear	*Ursus americanus*
		Brown bear	*Ursus arctos*
		Sun bear	*Ursus malayanus*
		Polar bear	*Ursus maritimus*
		Asiatic black bear	*Ursus thibetanus*
		Sloth bear	*Ursus ursinus*
Chiroptera	Miniopteridae	Little bent-winged bat	*Miniopterus australis*
		Common bent-winged bat	*Miniopterus schreibersii*
	Pteropidae	Straw-colored fruit bat	*Eidolon helvum*
Cingulata	Dasypodidae	Southern long-nosed armadillo	*Dasypus hybridus*
		Nine-banded armadillo	*Dasypus novemcinctus*
Eulipotyphla	Talpidae	Siberian mole	*Talpa altaica*
Pilosa	Myrmecophagidae	Giant anteater	*Myrmecophaga tridactyla*
FACULTATIVE			
Eulipotyphla	Soricidae	Eurasian water shrew	*Neomys fodiens*
		Common shrew	*Sorex araneus*
		Pygmy shrew	*Sorex minutus*
Rodentia	Cricetidae	Red tree vole	*Arborimus longicaudus*
		Small vesper mouse	*Calomys laucha*
		Northern collared lemming	*Dicrostonyx groenlandicus*
		Field vole	*Microtus agrestis*
		Bank vole	*Myodes glareolus*
		Northern grasshopper mouse	*Onychomys leucogaster*
		Cotton mouse	*Peromyscus gossypinus*
		White-footed mouse	*Peromyscus leucopus*
		Deer mouse	*Peromyscus maniculatus*
		Pinyon mouse	*Peromyscus truei*
		Campbell's hamster	*Phodopus campbelli*
		Dzhungarian hamster	*Phodopus sungorus*
		Chiriqui brown mouse	*Scotinomys xerampelinus*
	Muridae	Lesser short-tailed gerbil	*Gerbillus simoni*
		Common water rat	*Hydromys chrysogaster*
		Natal mastomys	*Mastomys natalensis*
		Sundevall's jird	*Meriones crassus*
		Shaw's jird	*Meriones shawi*
		Mongolian gerbil	*Meriones unguiculatus*
		House mouse	*Mus musculus*
		Spinifex hopping mouse	*Notomys alexis*
		Fawn hopping mouse	*Notomys cervinus*
		Sandy island mouse	*Pseudomys hermannsburgenesis*
		New holland mouse	*Pseudomys novaehollandiae*
		Bush rat	*Rattus fuscipes*
		Brown rat	*Rattus norvegicus*
		Indian gerbil	*Tatera indica*
MARSUPIALIA			
Dasyuromorphia	Dasyuridae	Brown antechinus	*Antechinus stuartii*
Diprotodontia	Acrobatidae	Feathertail glider	*Acrobates pygmaeus*
		Feathertail possum	*Distoeuchurus pennatus*
	Burramyidae	Western pygmy possum	*Cercartetus concinnus*
		Little pygmy possum	*Cercartetus lepidus*
		Eastern pygmy possum	*Cercartetus nanus*
	Macropodidae	Banded hare wallaby	*Lagorchestes fasciatus*
		Western hare wallaby	*Lagorchestes hirsutus*
		Agile wallaby	*Macropus agilis*
		Black-striped wallaby	*Macropus dorsalis*
		Tammar wallaby*	*Macropus eugenii*
		Eastern grey kangaroo	*Macropus giganteus*
		Western brush wallaby	*Macropus irma*
		Parma wallaby	*Macropus parma*
		Pretty-faced wallaby	*Macropus parryi*
		Common wallaroo	*Macropus robustus*
		Red-necked wallaby*	*Macropus rufogriseus*
		Red kangaroo	*Macropus rufus*
		Bridled nailtail wallaby	*Onychogalea fraenata*
		Allied rock wallaby	*Petrogale assimilis*
		Narbarlek	*Petrogale concinna*
		Black-footed rock wallaby	*Petrogale lateralis*
		Brush-tailed rock wallaby	*Petrogale pencillata*
		Prosperine rock wallaby	*Petrogale persephone*
		Purple-necked rock wallaby	*Petrogale purpureicollis*
		Yellow-footed rock wallaby	*Petrogale xanthopus*
		Quokka	*Setonix brachyurus*
		Red-bellied pademelon	*Thylogale billardierii*
		Red-necked pademelon	*Thylogale thetis*
		Swamp wallaby	*Wallabia bicolor*
	Potoroidae	Rufous bettong	*Aepyprymnus rufescens*
		Tasmanian bettong	*Bettongia gaimardi*
		Burrowing bettong	*Bettongia lesueur*
		Brush-tailed bettong	*Bettongia penicillata*
		Northern bettong	*Bettongia tropica*
		Gilbert's potoroo	*Potorous gilbertii*
		Long-nosed potoroo	*Potorous tridactylus*
	Tarsipedidae	Honey possum	*Tarsipes rostratus*

*Species with both obligate and facultative forms of embryonic diapause. Data compiled from references (Selwood, 1980, Renfree, 1981, Gilbert, 1984, Nelson and Goldstone, 1986, Hodara *et al.*, 1989, Sandell, 1990, Huang *et al.*, 1993, Atkinson, 1997, Newkirk *et al.*, 1997, Smith, 1998, Fisher, 1999, Johnson and Delean, 1999, Crocker *et al.*, 2001, Johnson and Delean, 2002, Courtenay and Friend, 2004, Thom *et al.*, 2004, Delean, 2007, Zhang *et al.*, 2009, Knott *et al.*, 2013)

an increased understanding of the embryo-uterine dialogue has implications not only for women with recurrent miscarriages but also in improving the success rate of embryo transfers resulting from assisted reproductive technologies.

Types of embryonic diapause: obligate, facultative or both

The concepts of obligate and facultative diapause have been chronicled in the reviews that have appeared over the previous 20 years (Mead, 1993; Renfree and Shaw, 2000; Lopes *et al*., 2004). Obligate diapause characterizes each gestation of a species, while facultative diapause is related to some condition, usually lactational or metabolic stress that, when relieved, allows implantation to occur. Lactational diapause is common in rodent species that have a postpartum estrus and in marsupials where the ovarian cycle and inter-uterine embryo development concur in duration. Some marsupial species display a combination of the two types of diapause, where lactational delay is further extended by seasonal photoperiodic cues (Renfree and Shaw, 2000). The model species described below illustrate the similarities and differences between the facultative and obligate manifestations of diapause.

Embryonic diapause in model species

Rodent facultative model

The best studied models of diapause are the rodents, in particular the mouse and the rat. There appears to be no evidence of obligate diapause in these species, rather facultative diapause, resulting from the metabolic stress of lactation, is the most common form. The length of the delay in both mice and rats correlates with the number of offspring in the suckling litter (Pritchett-Corning *et al*., 2013).

Mice display estrus immediately following parturition and will then mate. The consequent embryos enter the uterus at the morula stage on the third day after mating (day 1 = vaginal plug) and have become blastocysts by day 4, comprised of 35 to 40 cells (Harper, 1982; McLaren, 1982). Timely achievement of this state of development requires adequate progesterone secretion from the corpus luteum (Zhang and Murphy, 2013). If metabolic stress is present, the embryo then enters diapause. Over the next 72 hours it emerges from the lysed zona pellucida and continues proliferation to approximately 130 cells, and this complement of cells persists during diapause (McLaren, 1968; Fig. 1). During diapause, the blastocysts are evenly spaced and located in uterine crypts formed from luminal closure of the uterus. Apposition of the embryos also occurs with the embryonic pole aligning with the antimesometrial epithelium but the subsequent events of implantation do not occur (Psychoyos, 1973; Nilsson, 1974). The cellular mechanisms that suspend development of the mouse embryo are not well known. An interesting hypothesis is that gene expression is attenuated by microRNA (miRNA) degradation of transcripts during diapause, preventing translation of mRNA to proteins essential to continued development. This concept is supported by the recent observation that some 38 miRNAs are more abundant in the blastocyst in diapause, relative to its activated counterpart (Liu *et al*., 2012). Overexpression of one of these miRNAs, *Let-7a*, in mouse embryos prevents implantation (Liu *et al*., 2012). Targets of *Let-7a* include several genes that regulate cell proliferation (Gurtan *et al*., 2013), consistent with the view that there is depletion of specific elements required for progression beyond the blastocyst stage.

In spite of markedly lower protein synthesis and carbohydrate metabolism in dormant mouse blastocysts (Weitlauf, 1974), the needs for embryo survival are met. Recent studies have revealed a potential mechanism by which the metabolic requirements are supplied to the mouse embryo during diapause (Lee *et al*., 2011). These authors show that dissolution of a small number of cells by autophagy allows for the recycling of vital cell nutrients in the cells of the embryo.

Reactivation of the blastocyst from diapause is very rapid in the mouse and is characterized by increases in DNA, RNA and protein synthesis, cell number and metabolism within 4-16 h after the resumption of development, followed closely by the initiation of implantation (Yoshinaga and Adams, 1966; Paria *et al*., 1993; Das *et al*., 1994; Spindler *et al*., 1996). A recent proteomic analysis, remarkable in its investigation of 6000 embryos, identified numerous factors that were differentially expressed between the diapause and activated blastocyst (Fu *et al*., 2014). Not unexpectedly, elements essential to aerobic glycolysis and a wide range of factors related to chromatin remodeling and proliferation were upregulated with reactivation. Glutathione metabolism, a process associated with cell protection from reactive oxygen species damage, was increased with reactivation. Further analysis of this massive database is expected to provide new insights into the process of diapause.

Early studies demonstrated that, in both mice and rats, diapause could be terminated by administration of estrone (Krehbiel, 1941).

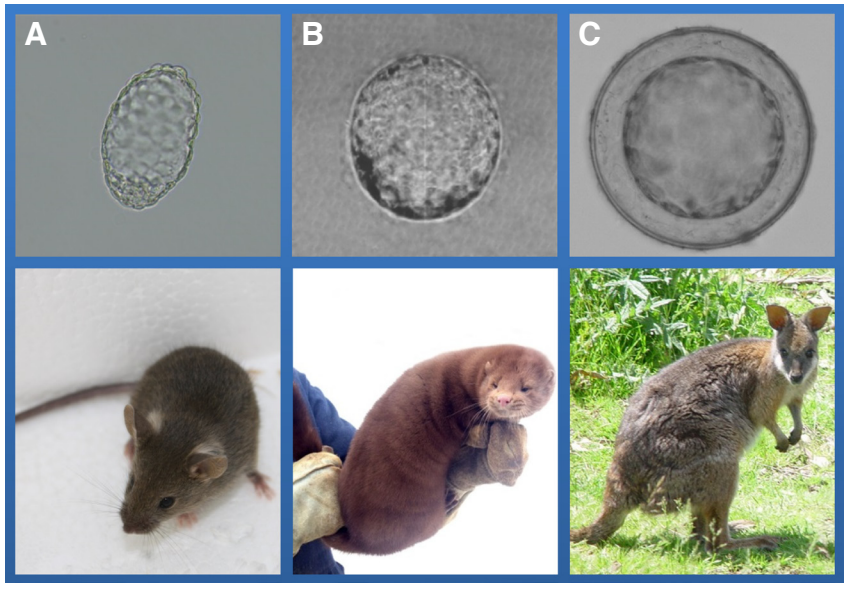

Fig. 1. Diapause embryos from the three model species. (A) *Mouse blastocyst containing an inner cell mass, with the zona pellucida already shed.* **(B)** *Mink blastocyst containing an inner cell mass and surrounded by a trilaminar capsule.* **(C)** *Wallaby blastocyst with no inner cell mass, surrounded by three extracellular layers; a zona pellucida, a mucoid layer and a shell coat.*

It was later shown that an ovarian surge of estradiol secretion during late morning of the fourth day following mating is essential to induction of implantation (McCormack and Greenwald, 1974). Much of what we know about rodent implantation can be attributed to use of an experimentally induced diapause model, the ovariectomized, progesterone-treated mouse or rat, in which implantation can be predictably induced by a single administration of estrogen (Cha *et al.*, 2013).

The ovarian compartment or cell type from whence this estrogen is secreted remains to be clarified. Furthermore, there appears to be no modern investigation of the upstream regulation of the nidatory estrogen surge in rodents. Treatment of hypophysectomized rats with luteinizing hormone (Macdonald *et al.*, 1967) resulted in successful implantation, while passive immunization with anti-LH antiserum in early gestation prevented this event (Maneckjee and Moudgal, 1975). Beyond these observations, little is known.

The estrogen of ovarian provenance that effects implantation in the mouse uterus acts through the estrogen receptor-α (Esr1) and estrogen receptor-β (Esr2). Esr1 is expressed in the uterine glands, endometrial epithelium and endometrial stroma during the period that coincides with the ovarian estrogen signal (reviewed in Vasquez and DeMayo, 2013). The role of Esr2 is less well defined, as germline deletion of the gene does not prevent implantation (Couse and Korach, 1999).

The progesterone receptors (Pgr) in the mouse uterus are essential for embryo transport prior to implantation and are clearly involved in preparation of the uterus for embryo implantation (Cha *et al.*, 2012). The Pgr A isoform is the major functional form in the mouse uterus, and is found in the stromal, glandular and epithelial compartments of the endometrium (Vasquez and DeMayo, 2013). At the time of initiation of implantation, Pgr expression ceases to occur in the luminal and glandular epithelia, and remains only in the stroma, allowing for the estrogen-induced proliferation of the epithelium that precedes implantation (Vasquez and DeMayo, 2013). In the absence of the estrogen signal, i.e. the delay state, Pgr expression persists in the glandular and epithelial compartments, presumably contributing to embryo quiescence.

Mustelid carnivore obligate model

Obligate diapause is a widespread phenomenon among carnivores, with numerous examples in the orders *Ursidae, Mephitidae, Mustelidae, Otariidae* and *Phocidae* (Table 1). Two models have been studied in some detail, the Western spotted skunk *(Spilogale gracilis)* and the American mink *(Neovison vison).* The former species mates in the autumn with implantation and parturition occurring in late spring, with embryos in diapause for approximately 200 days (Mead, 1981). The mink, on the other hand, breeds in early to mid-March in the Northern Hemisphere, after which the embryos undergo a brief delay followed by implantation and a post-implantation gestation of 30-31 days (Murphy, 1992).

The early embryology is nearly identical between the two carnivore species, in that blastocysts develop at approximately six days after mating. During diapause in the mink, the blastocysts remain clustered in the anterior portion of the uterus with migration and spacing only occurring once reactivation has occurred. The carnivore blastocyst differs from that of the mouse in two important features, firstly, the number of cells in the embryo is substantially greater and more variable, usually reported to range from 250 to 500 cells (Fig. 1). Secondly, the embryo remains encapsulated in the zona pellucida of the oocyte which has become trilaminar, presumably due to coatings invested during the transit through the reproductive tract (Enders *et al.*, 1986). In the skunk, there is a minor increase in the number of trophoblast cells during the lengthy period of diapause (Mead, 1981), reported to be due to endoreduplication and cell division without nuclear envelope breakdown (Isakova and Mead, 2004). A fraction of the cells of the mink embryo demonstrate DNA synthesis during diapause (Llerena-Vargas, 2011). In both species there is rapid expansion of the blastocyst and a rapid increase in both cell replication and protein synthesis associated with the reactivation of embryo development (Enders *et al.*, 1986; Desmarais *et al.*, 2004).

The increasing photoperiod following the vernal equinox is the proximal stimulus for the termination of dormancy and the consequent escape of the embryo from diapause in these carnivores (Murphy and James, 1974). It is mediated via the pineal gland by a decrease in the duration of the nocturnal secretion of melatonin (reviewed in Lopes *et al.*, 2004). Increasing photoperiod translates into increased secretion of prolactin from the pituitary (Murphy, 1983), and can be prevented by dopamine agonist treatment (Papke *et al.*, 1980), hypophysectomy (Murphy *et al.*, 1981) and chronic melatonin treatment (Murphy *et al.*, 1990). All three treatments abrogate implantation. Termination of diapause and precocious implantation can be induced by the administration of prolactin (Papke *et al.,* 1980; Murphy *et al.,* 1981). Reactivation of the embryo is first visible three days later in the form of embryo expansion, with implantation occurring around 13 days after initiation of prolactin treatment (Desmarais *et al.*, 2004).

The corpora lutea in both the mink and skunk develop following ovulation, but cell volumes actually decline from their initial size at differentiation (Mead, 1981; Douglas *et al.*, 1998a). Progesterone synthesis occurs, as reflected in circulating concentrations, but at a much reduced level relative to post-implantation gestation (Papke *et al.*, 1980; Mead, 1981; Douglas *et al.*, 1998a). Increased circulating prolactin following the equinox induces expression of both prolactin and LH receptors in the mink corpus luteum, and these two hypophyseal factors then drive progesterone synthesis (Murphy and Rajkumar, 1985; Douglas *et al.*, 1998a). A peculiarity of the mink corpus luteum of diapause is that the cells are not terminally differentiated by luteinization as in other species, but retain their capacity to divide (Douglas *et al.*, 1998b). Implantation requires secretion of an as yet unknown factor from the corpus luteum (Murphy *et al.*, 1983). Parallel studies in the domestic ferret indicate that this factor is proteinaceous (Mead *et al.*, 1988), a viable candidate is glucose-6-phosphate isomerase, functioning as a cytokine (Schulz and Bahr, 2003).

The endocrine maintenance of the uterine environment hospitable to the embryo in diapause and the modifications of this environment that are permissive to implantation have yet to be well explored. Multiple studies have shown that implantation is preceded by a rapid increase in circulating progesterone from the reactivated corpus luteum (Stoufflet *et al.*, 1989; Murphy, 2012). Prolactin binds to membrane preparations from the anestrous mink uterus (Rose *et al.*, 1983). Prolactin receptor binding to the uterus increases substantially during diapause compared to the anestrous condition, then declines following implantation (Rose *et al.*, 1986). Analysis of prolactin receptor abundance by immunoblotting corroborates binding assay observations (Llerena-Vargas 2011). Direct effects of prolactin in the uterus of the mink remain

to be demonstrated.

The paradigm employed successfully in the mouse, i.e. estrogen administration against a background of progesterone, does not induce implantation in the mink. Progesterone and estrogen receptor binding to the mink uterus have been explored in anestrous mink, and the results indicated that suppression of prolactin secretion increased progesterone, but not estrogen receptor populations (Slayden and Stormshak, 1992). Nuclear estrogen receptor abundance, as established by binding assays, was greater in pregnant, post-implantation mink uteri compared to the anestrous condition (Patnode and Curtis, 1994). The same authors demonstrated progesterone receptors to be present in the post-implantation uterus, but no comparisons between diapause and later gestation were made. Progesterone receptors, along with both ESR1 and ESR2 are present in the uterus of the California sea lion *(Zalophus calforianus)*, a pinniped carnivore (Colegrove *et al*., 2009). Semiquantitative analysis by immunohistological staining suggests that both are substantially lower in the endometrium during diapause, relative to post-implantation gestation. No variation was evident in progesterone receptor abundance in the surface and glandular epithelium between diapause and later gestation in this species (Colegrove *et al*., 2009). Clearly, further investigation of steroid receptors during gestation and diapause in carnivores is warranted.

Marsupial combined facultative and obligate model

In marsupials, embryonic diapause is a common reproductive strategy, particularly amongst the Macropodidae (Table 1), where there are only three known exceptions in which it is absent, the western grey kangaroo (*Macropus fuliginosus*), musky rat kangaroo (*Hypsiprymnodon moschatus*) and Lumholtz's tree kangaroo (*Dendrolagus lumholtzi*) (Tyndale-Biscoe, 2005). The control of embryonic diapause in marsupials has been best studied in the tammar wallaby (*Macropus eugenii),* which, in contrast to the mouse and mink, is monovular and undergoes both facultative and obligate forms of embryonic diapause (reviewed in Renfree and Shaw, 2000). Facultative diapause in the wallaby occurs after mating at the postpartum estrus, and is induced by the suckling stimulus of the newborn young in the pouch. Tammar wallabies give birth to single, precocious young in late January in the southern hemisphere, which then completes its development in the pouch, emerging around late September. Mating occurs following parturition, and, if the pouch young is lost before May, the single embryo in diapause will reactivate and be born around 27 days later. If however the pouch young is lost after this time, the embryo will remain in a seasonally-induced obligate diapause until late December (after the summer solstice). Thus, the wallaby embryo can remain in diapause for as long as eleven months.

The wallaby blastocyst forms after 6 days and, in contrast to Eutherian species, is a unilaminar structure of around 80 cells with no inner cell mass, and with no sign of an embryonic disc appearing until around day 9 after reactivation (Fig. 1; Renfree, 1994; Frankenberg *et al*., 2013). Similar to the carnivores, the wallaby blastocyst remains enclosed in its zona pellucida during diapause but there are also an additional two layers, a mucin layer and a shell coat, both laid down during passage through the reproductive tract (Fig. 1). During diapause, the single blastocyst remains free floating in the uterus with no observable mitosis or cell growth, but it maintains a basal metabolism and low levels of RNA and protein synthesis (Moore, 1978; Tyndale-Biscoe, 1978; Thornber *et al*., 1981; Shaw and Renfree, 1986; Tyndale-Biscoe and Renfree;1987, Spindler *et al*., 1998; Spindler *et al*., 1999a). Reactivation of the blastocyst from diapause is characterized by a resumption of the cell cycle and increased metabolism.

Although tammar wallabies undergo both facultative and obligate diapause, endocrine cues regulating either version result in inhibition of the immature corpus luteum by prolactin secreted from the pituitary (Hinds, 1989; Hinds and Tyndale-Biscoe, 2013). During facultative diapause, the suckling of the pouch young sends a neural signal to the hypothalamus, whilst during obligate diapause, photoperiodic signals are transduced by melatonin secretion from the pineal gland (Renfree, 1979; Tyndale-Biscoe *et al*., 1986). Reactivation from embryonic diapause in the wallaby requires removal of the prolactin-induced inhibition of the corpus luteum for a minimum of three days by either removal of the pouch young or by a decreased photoperiod accompanied by a change in the length of the daily episode of melatonin secretion (Hinds and Tyndale-Biscoe, 1982; Hinds *et al*., 1983; Shaw and Renfree, 1984; Hinds, 1989). Diapause can be experimentally terminated in the wallaby by hypophysectomy or by treatment with either the dopamine agonist, bromocriptine during lactational quiescence, or by melatonin during seasonal quiescence (Hearn, 1974; Tyndale-Biscoe and Hinds, 1984; 1992). Nonetheless, quiescent wallaby blastocysts are not reactivated by bilateral ovariectomy (Tyndale-Biscoe and Hearn, 1981). Reactivation from diapause occurs slowly in the wallaby with the first signs that the blastocyst has reactivated at day 4, followed by embryo expansion at day 8, with shell coat rupture and attachment to the uterine wall occurring around 18 days after reactivation (Renfree and Tyndale-Biscoe, 1973; Denker and Tyndale-Biscoe, 1986; Shaw and Renfree, 1986; Spindler *et al*., 1998; Spindler *et al*., 1999b).

At entry into diapause, the corpus luteum is still maturing, and progesterone levels are basal (Tyndale-Biscoe and Renfree, 1987). Prolactin secreted daily from the pituitary prevents the preimplantation progesterone pulse from occurring (Hearn, 1974; Tyndale-Biscoe and Renfree, 1987; Hinds, 1989). During diapause, high concentrations of prolactin receptors are present in the corpus luteum, levels of which halve at reactivation (Stewart and Tyndale-Biscoe, 1982). Nonetheless, prolactin does not have a luteotrophic role in the corpus luteum in marsupials and there is no evidence for luteinizing hormone binding to this tissue (Stewart and Tyndale-Biscoe, 1982; Bradshaw and Bradshaw, 2011). Removal of the prolactin inhibition is then followed by a pulse of progesterone around day 5 of reactivation. Both ESR and PGR are expressed in the wallaby endometrium at high concentrations during both diapause and early reactivation, but both decrease to relatively low levels after the pulse of progesterone (Renfree and Blanden, 2000). Both ESR1 and ESR2 isoforms exist in the tammar, but it is unknown whether there are any differences in their expression in the endometrium. Although both estradiol and progesterone can initiate reactivation in the wallaby, it can only be maintained by progesterone (Renfree and Tyndale-Biscoe, 1973; Fletcher *et al*., 1988).

Thus in the three model species, the sequence of endocrine events leading to diapause and reactivation are well defined, albeit variant. Although there are multiple mechanisms involved in diapause across mammals, all appear to exert their effects on the uterine environment. However, it is unclear how the uterine

environment acts on the blastocyst to control diapause or whether this process is conserved across species.

Commonalities in embryo implantation

Numerous elements of the initial implantation process appear conserved among mammals. The notion of a temporal period of uterine receptivity, known as the window of implantation, was established by asynchronous transfers of embryos into the uteri of females in various stages of pseudopregnancy (Psychoyos, 1986). The concept has proven valid in virtually all species studied (Yoshinaga, 1988). Among the constants for receptivity across species is the presence of luteal progesterone, and, in many species, ovarian or locally synthesized estrogens (Zhang *et al.*, 2013). Consistent with this, reactivation from diapause also requires the presence of steroid hormones and a lack of uterine receptivity has been suggested to be a factor in the induction of embryonic diapause (Paria *et al.*, 1993). A second generality of receptivity is that it engenders alteration of anti-adhesive elements, principally glycoproteins found on the endometrial epithelial surface, that have the capacity to inhibit implantation (Bazer *et al.*, 2010). Attachment of the trophoblast to the extracellular matrix of the endometrial epithelium is a progressive process, first involving carbohydrate ligand binding, then employing factors that include the selectins, trophonin and heparin-binding epidermal growth factor (HBEGF) (Fukuda and Sugihara, 2008).

The dialogue between the embryo and uterus, to date only partially understood, is an essential element of both embryonic diapause and the implantation process in mammals. In polytocous species, embryos become spaced along the uterine horn. It is hypothesized that the mouse embryo secretes factors, perhaps proteins of the wingless (Wnt) family that activate Wnt signaling in the uterus with effects on the myometrium, that may then, in turn, affect the spacing of the embryos (Mohamed *et al.*, 2005). Targeted deletion of cytosolic phospholipase-A2α disrupts embryo spacing, indicating that lipid signaling plays a role in this process (Song *et al.*, 2002).

There appear to be other common factors of uterine provenance essential for implantation across species, in particular, the cytokines such as interleukin-1β (IL1b)(Bourdiec *et al.*, 2013), leukemia inhibitory factor (LIF), and growth factors including bone morphogenic protein-2 (BMP2), fibroblast growth factors (FGF) and HBEGF, among others (Cha *et al.*, 2012). In the mouse, the binding of endometrial Hbegf to its receptor, Erbb4 on the blastocyst, coordinates the initiation of implantation, a mechanism consistent with observations in humans (Yoo *et al.*, 1997; Leach *et al.*, 1999; Chobotova *et al.*, 2002). Further candidates are the signaling molecules of the WNT family (Chen *et al.*, 2009; van der Horst *et al.*, 2012). The muscle segment homeobox-1 (Msx1) is essential for uterine receptivity in the mouse (Daikoku *et al.*, 2011). Msx1 is an upstream regulator of the *Wnt* genes and its targeted disruption in the uterus disrupts Wnt signaling patterns, altering the state of the uterine epithelium in the mouse (Nallasamy *et al.*, 2012). Uterine polyamines also appear to be essential, at least in carnivore and rodent species (Lefevre *et al.*, 2011c).

Knowledge of embryonic signals in the implantation process is sparse, prostaglandins of the E-series are candidates (Lopes *et al.*, 2007). However, many of the above factors are also present in the blastocyst. Currently, limited evidence is available confirming which of the factors active during implantation are involved in the control of embryonic diapause. A more profound understanding of embryo-uterine communication is key to elaboration of the mechanisms of embryonic diapause.

The uterus dictates diapause

In the mouse, reactivation of the uterus after diapause is regulated by ovarian steroid hormones, and the induction of a favorable uterine environment is required for subsequent development of the blastocyst (Paria *et al.*, 1993). However, the *in vitro* addition of steroid hormones directly to mouse embryos in diapause does not result in their reactivation (Weitlauf, 1974).

Embryo transfers of blastocysts to delay-state uteri in rodents (Weitlauf and Greenwald, 1968) and reciprocal transfer of embryos from a species displaying obligate diapause, the mink, to a species without evidence of diapause, the domestic ferret (Chang, 1968) have defined the uterus as the regulator of developmental arrest. Similarly, mink blastocysts in diapause resumed development when co-cultured with conspecific uterine cell lines, and wallaby blastocysts in diapause are able to reactivate and develop normally when transferred to a reactivated uterus (Tyndale-Biscoe, 1970; Moreau *et al.*, 1995). Wallaby blastocysts displaying delay in their metabolic reactivation were found to be from mothers with a low endometrial metabolism, indicating a failure of the maternal system to reactivate (Spindler *et al.*, 1998). Since both the wallaby and mink blastocyst are surrounded by multiple acellular layers and do not implant until a number of days after reactivation, factors controlling diapause must reach the embryo via the uterine secretions (Renfree, 1973; Shaw and Renfree, 1986; Renfree and Shaw, 2000).

The notion of uterine regulation of diapause is further strengthened by the remarkable recent findings that the sheep blastocyst can be induced to undergo developmental arrest if transferred to the uteri of pseudopregnant mice, and can then, if transplanted back to the uterus of the ewe, produce normal lambs (Ptak *et al.*, 2012).

As noted above, numerous factors secreted by the uterus that are essential to the implantation process are common to multiple species, and their expression, or lack thereof, is expected to be a mechanism by which the uterus dictates diapause. The first sign of reactivation from diapause in the mouse is the detection of Hbegf in the endometrium, 6-7 hr before implantation commences but only adjacent to the blastocyst, (Das *et al.*, 1994). We recently showed that Msx1 or Msx2, transcriptional regulators of some uterine implantation factors, persist in the uterus across three species of widely differing taxa, mice, mink and wallabies, during embryonic diapause (Cha *et al.*, 2013). Together, the observation of uterine control and conserved regulation indicates common mechanisms were established during the evolution of diapause. Furthermore, the findings of Ptak *et al.,* (2012) in the sheep suggest that the potential for diapause is not restricted to the species where it is known to occur.

The mechanisms by which the uterus induces and maintains developmental arrest remain to be established. Some evidence points to an inhibitory effect of the uterine environment that inhibits embryo development during diapause. Incubation of rabbit blastocysts with the protein fraction of flushings from the mouse uterus in the diapause state inhibits embryonic DNA synthesis (Weitlauf, 1978). In the mouse, levels of the endocannabinoid, anandamide

appear to play a significant role in regulating reactivation of the blastocyst from embryonic diapause (Wang *et al*., 2003). Whilst high levels of anandamide maintain diapause, low levels can activate the blastocyst. In addition, anandamide binds to the cannabinoid receptors, CB1 and CB2. The levels of CB1 are up-regulated in the blastocyst trophectoderm during diapause and rapidly down-regulated at reactivation (Wang *et al*., 2003). Hence, high levels of anandamide may act as an inhibitory factor in the mouse. Although the mouse embryo is able to reactivate its metabolism *in vitro*, it is not "implantation competent" (Weitlauf, 1974; Paria *et al*., 1993; Spindler *et al*., 1998; Spindler *et al*., 1999a). Furthermore, incubation of mouse blastocysts with wallaby uterine exudates collected during diapause did not completely cause the mouse embryo to enter into diapause and did not maintain diapause *in vitro* (Spindler *et al*., 1999a).

The contrasting view is that during diapause, the uterine factors essential for continued embryogenesis are lacking, thus resulting in a developmental arrest. Although the uterus of the mouse has fewer endometrial glands than found in the other model species, the glands are active in secretion during the peri-implantation period (Given and Enders, 1980). Termination of experimental diapause in the mouse by estrogen treatment induces changes that parallel those seen during implantation (Given and Enders 1978), suggesting glandular secretions essential to implantation are not present during diapause. In the wallaby during diapause, uterine secretions are low and the uterus remains quiescent (Renfree, 1973; Shaw and Renfree, 1986; Tyndale-Biscoe and Renfree, 1987; Spindler *et al*., 1998). Furthermore, dormant blastocysts from both the wallaby and the mink do not reactivate when cultured *in vitro* (Moreau *et al*., 1995; Renfree and Shaw, 2000). One potential candidate for a missing factor is LIF, which is essential for embryonic stem cell proliferation (Zhao *et al*., 2012) and for implantation in the mouse (Hondo and Stewart, 2005). LIF expression in the uterine epithelium and glands appears at the termination of diapause in the mouse, mink and the wallaby (Bhatt *et al*., 1991; Stewart *et al*., 1992; Song *et al*., 1998;Renfree, personal communication). In the mouse, LIF expression appears to be under estrogenic control and can replace the estrogen injection to induce reactivation (Chen *et al*., 2000).

However, it is unlikely that reactivation from embryonic diapause is the result of one particular signal, but a combination of a range of factors, each with a specific downstream function. Among the best recent candidates for uterine factors that are required to terminate diapause are the polyamines, known to be involved in diverse reproductive functions in mammals (Lefevre *et al*., 2011c). Polyamine synthesis is rate limited by the ornithine decarboxylase-1 (ODC1) enzyme (Lefevre *et al*., 2011c). As noted above, an episode of ovarian estrogen secretion is the key event in termination of mouse diapause. It has been shown that there is an increase in the expression of not only *Odc1*, but also other genes that modulate polyamine availability, *Sat1, Samdc, Sms* and *Smox*, at the time of estrogen-induced reactivation of the mouse blastocyst (Zhao *et al*., 2008). Implantation sites in the mouse display elevated levels of expression of Odc1 and polyamines (Zhao *et al*., 2008). Blockade of Odc1 conversion of ornithine to the polyamine putrescine by the suicide inhibitor difluoromethyl ornithine (DFMO) prevents implantation in the mouse (Zhao *et al*., 2008) and reversibly arrests development in mink (Lefevre *et al*., 2011b) and mouse (Zwierzchowski *et al*., 1986) embryos. Expression of ODC1, along with the polyamine availability genes (Lefevre *et al*., 2011a) and uterine content of the polyamine putrescine (Lefevre *et al*., 2011b), are upregulated at the termination of diapause in the mink (Murphy, 2012).

Although polyamines have pleiotropic actions in cellular systems, few molecular targets have been defined (Zhao *et al*., 2012). The mechanisms by which their absence or paucity induces or maintains diapause remain to be determined, but, given the developmental arrest of diapause, regulation of cell proliferation is an obvious candidate. Polyamines act as supercations at physiological pH, thus interacting with polyanions in the cell, including nucleic acids, proteins and phospholipids (Wallace *et al*., 2003). Polyamines stabilize DNA structure and promote chromatin remodeling and transcription (Wallace *et al*., 2003). Polyamine synthesis is associated with the cell cycle, with synthetic peaks at the G1-S transition and during the S-phase of the cycle (Alm and Oredsson, 2009). In neuroblastoma cells, inhibition of polyamine synthesis leads to accumulation of the cell cycle inhibitors and arrest of the cell cycle in the G0/G1 phase (Koomoa *et al*., 2013). The evidence, albeit limited, indicates that diapause, at least in the mouse, is also due to arrest prior to the S-phase of the cell cycle (reviewed in Lopes *et al*., 2004). Microarray analysis comparing dormant with reactivated mouse embryos showed increased expression of cell cycle inhibitory factor genes in the diapause condition (Hamatani *et al*., 2004). An attractive, if simplistic hypothesis is that, in the absence of polymines, the cells of the diapause embryo remain in a state of interphase.

The embryo plays a proactive role in its own development

Although it appears that uterine factors control the events of embryonic diapause, the blastocyst itself also has a role in determining its own development. In many eutherian species, embryo signaling ensures maternal recognition of pregnancy by maintaining a functional corpus luteum, although the factor involved can differ, depending on the species (reviewed in Bazer *et al*., 2010). In the mouse uterus at decidualization, approximately 10% of all genes expressed in the endometrium are differently expressed in the presence of an embryo (Kashiwagi *et al*., 2007). Studies have demonstrated pinocytic activity in the rat uterus at the time of implantation, indicating uptake of substances from the lumen (Enders and Nelson 1973), and providing a mechanism for transfer of embryonic signals to the endometrium. The level of pinocytosis, as determined by morphological methods, is lower when the embryos are in diapause (Enders and Nelson 1973). It is not clear whether the material taken up is of embryonic provenance, but this observation, along with the information cited above suggests that the embryo takes a proactive role in preparing the maternal environment to support its own development. Mouse embryos can survive in simple, defined media, indicating a certain autonomy from the uterine environment, because they are believed capable of producing autocrine factors to enhance their own development (Whitten, 1957). It is therefore possible that, during diapause, the absence of paracrine signaling from the uterus down-regulates this autocrine production by the embryo.

In the mouse there are over 200 genes differentially expressed between the dormant and activated blastocyst, 149 of which are upregulated at reactivation (Hamatani *et al*., 2004). One of these, Hbegf, is the only ligand significantly upregulated in the blastocyst at reactivation, and is able to induce its own expression in the en-

dometrium (Hamatani *et al.*, 2004). It would therefore appear that the embryo can function as an active unit with its own molecular program of cell growth and differentiation (Paria *et al.*, 2002; Dey *et al.*, 2004; Dey and Lim, 2006).

Diapause: a common mechanism or convergent evolution?

Given that we were not present during the evolutionary trajectory that resulted in the establishment of the reproductive mechanisms of extant species, suppositions on the evolution of diapause cannot be confirmed. Two divergent views can be considered (Table 2). It has been theorized by Sandell (1990) that diapause evolved independently multiple times, in both its facultative and obligate manifestations. This conclusion is based primarily on the occurrence or non-occurrence of diapause in congeneric species such as the ermine (*Mustela ermina*) and long-tailed weasel (*Mustela frenata*), both diapause species, and the least weasel (*Mustela nivalis),* a species that displays no diapause. A classic example is the spotted skunk, where the western species (*Spilogale gracilis*) displays obligate diapause and its eastern counterpart, (*Spilogale putorius*), does not (Mead, 1993). A second argument for convergent evolution is that the trait is found in a wide variety of species, including armadillos, at least one mole, three species of bats and an ungulate, the roe deer (Table 1) that have a wide divergence of placentation, post-implantation embryogenesis and other reproductive strategies. As can be seen in the model species, the diversity of regulatory mechanisms further argues for independent evolution of diapause. Prolactin can maintain diapause (marsupials), terminate diapause (mustelids) or have no effect (rodents). The ovaries are essential for implantation in the model species, but, remarkably, in the nine-banded armadillo (*Dasypus novemcinctus*), implantation of blastocysts in diapause is induced by ovariectomy, occurring 18-29 days after removal of the ovaries in the absence of exogenous hormone treatment (Buchanan *et al.*, 1956).

There is evidence for the contrary view, i.e. that diapause evolved but a few times, or even only once, and that control mechanisms have evolved to exploit the trait when it provides reproductive advantage to the species. The facultative condition argues for this possibility, in that an embryo can undergo diapause or not, depending on metabolic stress on the dam. In the absence of a pouch young, the wallaby embryo will not undergo diapause and its gestation length is shortened as a result (reviewed in Tyndale-Biscoe and Renfree, 1987). This concept is further supported in the context of obligate diapause, in that development was arrested at the blastocyst stage in ferret, a non-diapause species, when these embryos were transplanted to the uterus of the mink (Chang, 1968). Furthermore, delay in implantation was induced in ferrets by manipulation of the ovarian hormonal milieu (Foresman and Mead, 1978).

The work of Ptak and colleagues (2012), whereby they successfully induced diapause in sheep embryos by placing them in the uterus of a mouse has provided a new perspective on the question. The same laboratory has apparently repeated the finding using two other non-diapause species, the cow and the rabbit (alluded to but unpublished by Ptak *et al.*, 2013). These experiments indicate that embryos of non-diapause species can survive in the blastocyst state for an extended period of time, and be reactivated in a pattern similar to that seen at termination of delayed implantation in the mouse. The experimental paradigm of Ptak (2012) provides for the possibility of an in depth exploration of the embryo-uterine dialogue that will shed further light on the evolution of diapause.

TABLE 2

THE ORIGIN OF DIAPAUSE, CONVERGENT EVOLUTION *vs.* A SINGLE EVOLUTIONARY EVENT

Diapause evolved once	Diapause evolved multiple times
Facultative diapause-dormancy can be induced	Diapause is present in a wide variety of species with greatly differing implantation processes and placentation
Presence/absence in congeneric species	Wide variety of endocrine regulatory mechanisms
Induction of diapause in non-diapause species by transfer of embryos to the delay uterus	Differences in embryonic characteristics of diapause among species
Commonality of uterine regulatory mechanisms	
Always occurs at the blastocyst stage	

Pressing questions

While progress has been made in the understanding of embryonic diapause in recent years, a number of issues remain to be more satisfactorily resolved. The embryo-uterine dialogue that first suspends development and then allows it to recommence, requires further exploration. In particular it would be valuable to confirm whether the uterus directly inhibits development during diapause, or whether the arrest in development is due to deprivation of factors necessary for further embryogenesis. We know little about the mechanisms of suspension of the cell cycle or about the strategy employed by the embryo to survive at the very low metabolic rate of diapause. It is hoped that the rapidly evolving cell and molecular biology technology will provide the tools to answers these and other questions pertinent to diapause.

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