# Protection in the pouch: antimicrobial peptides in marsupials and monotremes

**Emma Peel[1¶], Elizabeth Jones[1¶], Katherine Belov[1]** and **Yuanyuan Cheng[1*]**

[1] Faculty of Veterinary Science, The University of Sydney, NSW 2006, Australia
[¶] These authors contributed equally to this work.
* Corresponding author

The production of antimicrobial peptides (AMPs) represents an ancient form of host defence against microbial pathogens and has been identified in both plants and animals. Among the many different antimicrobial peptides, cathelicidins and defensins are the two major families that have been intensively studied in mammals. These small AMPs are usually hydrophobic and highly charged, which enables them to selectively interact and kill a large range of bacterial, fungal or viral pathogens. The recent sequencing of several marsupial and monotreme genomes has led to the identification of AMPs in these divergent mammalian lineages.

Marsupials and monotremes last shared common ancestors with their eutherian counterparts over 160 million years ago. They provide a unique opportunity to gain insights into the evolution of immunity in mammals due to their unique reproductive characteristics. Young marsupials and monotremes are born immunologically immature. Their immune system develops outside the sterile confines of a uterus in the first few months of life, during which they face a broad range of pathogens in the pouch or the burrow. These unique features may have driven the observed gene family expansion within the cathelicidin and defensin gene families, resulting in a panel of broad-spectrum AMPs which can provide non-specific immunity to immunologically naïve young. We have shown that the peptides synthesised by the young themselves and the mammary glands of the mother have broad-spectrum activity against a variety of fungi and gram positive and gram negative bacteria, including several multi-drug resistant strains. In this article, we will briefly review the features of cathelicidins and defensins in eutherian mammals and then compare that with what we know about marsupial and monotreme AMPs. We propose that these peptides may provide novel therapeutics for emerging multi-drug resistant organisms.

**Keywords** antimicrobial peptide; cathelicidin; defensin; marsupial; monotreme

## 1. Marsupials and monotremes – the non-eutherian mammals

Marsupials and monotremes are subclasses of mammals with distinctive reproductive anatomy and physiology which separate them from eutherian (placental) mammals. Diverged from eutherians around 160 million years ago, marsupials and monotremes have evolved into more than 300 species distributed across Australia, America and Papua New Guinea [1]. The feature which commonly represents marsupials (e.g. kangaroos and koalas) is the female's pouch or marsupium, though the pouch is not fully developed in all species and can be rudimentary or even absent (e.g. the grey short-tailed opossum, *Monodelphis domestica*) [1]. Monotremes (echidnas and the platypus) differ further still: they are the only known oviparous mammals, with a reproductive tract closely resembling that of birds and reptiles [1]. After egg-laying, the newborns are incubated and raised either in a pouch, as seen in the short-beaked echidna (*Tachyglossus aculeatus*), or in a burrow, as seen in the platypus (*Ornithorhynchus anatinus*).

### 1.1. Short gestation and altricial young

Both marsupials and monotremes have characteristically short gestation periods, culminating in the birth of highly altricial young. In eutherians, the duration of gestation period varies greatly among species due to differences in body size, growth rate and reproductive cycles, but in general, eutherian young are born at a late developmental stage. In marsupials, the length of gestation varies from 15 days in opossums to 35 days in macropods, which is significantly shorter as compared to eutherian species with a similar body size [1]. Similarly, monotremes also have short gestation periods, with the length estimated to be 15-21 days in the platypus and 21-23 days in the short-beaked echidna [2].
Corresponding to the short gestation, the timing of foetal development and metabolic demands on the mother in marsupials and monotremes also differ significantly from that seen in eutherians. Eutherian mammals undergo extensive growth in-utero as the mother sustains the foetus through transfer of nutrients and oxygen across the placenta. In contrast, marsupials undergo the majority of development after birth with the mother providing sustenance through complex changes to the volume and composition of milk in different phases of growth [3]. As such, compared to their eutherian counterparts, marsupial newborns are small and underdeveloped with body weight as low as 200-400 mg. Even the largest of marsupials gives birth to young weighing only 0.003% of maternal body weight [1].

### 1.2. Neonatal immune protection against pathogenic challenges

One key feature that separates the development of non-eutherians and eutherian mammals is that, due to birth at such an early developmental stage, the neonates of marsupials and monotremes are immunologically naïve lacking differentiated lymphoid tissues and mature immune effector cells [4]. Unlike eutherians, which are protected by the sterile confines of the mother's uterus during immune system development, the highly immature marsupial and monotreme neonates are challenged by wide ranges of pathogens within the non-sterile environment of the pouch or burrow [5]. The mechanism underlying the immune protection of the immunologically naïve neonates is not fully understood, though several strategies may be involved.

Firstly, an important source of protection comes from the passive transfer of maternal antibodies in-utero and through the milk. Prenatal transfer of maternal antibodies has been documented in the tammar wallaby (*Macropus eugenii*), an Australian marsupial model species. Immunoglobulins were isolated from the foetal and neonatal serum prior to suckling [3]. However, similar experiments in the Virginia opossum (*Didelphis virginiana*) and the grey short-tailed opossum failed to isolate antibodies from the newborn serum [3]. The transfer of maternal immunoglobulins and defence-associated cells through the milk occurs in two phases [3]. The first is the colostral phase, which is produced at the time of birth similar to other mammals. Examination of the milk profile during this phase in the tammar wallaby showed high levels of neutrophils, macrophages and lysozyme [3, 4]. Lymphocytes were not present until later in lactation [4] although immunoglobulins such as IgG were found at low levels (0.8%) in the tammar wallaby, quokka (*Setonix brachyurus*) and brushtail possum (*Trichosurus vulpecula*) [6]. In the latter two species, uptake of immunoglobulins occurs until the young exits the pouch [3]. The second phase of maternal immune protection transfer through milk takes place around 100 days post-partum and serves to protect the young as it first exits the pouch [3].

Secondly, the innate immune system develops rapidly after birth, providing another line of defence. In the tammar wallaby, it is observed that the innate immune response is acquired within the first 30 days post-partum [4]. In the Virginia opossum, leukocyte populations undergo expansion in the first two weeks of development, giving rise to a leukocyte level four-fold higher than that in adults [7, 8]. Neutrophils are the predominant cell type in these early leukocyte populations and are fully mature by day 5, enabling responses to foreign organisms through phagocytosis and inflammation. However, in contrast to the early emergence of innate immune responses, there is a delay in adaptive immunity development due to the lack of primary lymphoid organs responsible for lymphocyte differentiation and maturation in the early stage. In the Virginia opossum, lymphocytes comprise only 4% of the expanded leukocyte population and are not present until 10-12 days after birth [7]. In the tammar wallaby, the cervical and thoracic thymuses, which are the key organs for T cell differentiation and selection, mature at day 21 and 30, respectively [4]. It is also estimated that lymph nodes, the major site of B cell production, do not mature until day 60 and day 90 in the tammar wallaby and the opossum, respectively [4].

In addition to these mechanisms, antimicrobial peptides (AMPs) are likely to play an essential role in protecting marsupial and monotreme young against pathogenic infections. The recent whole-genome sequencing of the tammar wallaby, grey short-tailed opossum and platypus has allowed discovery and characterisation of novel AMPs in these species [9-12]. Further functional studies have revealed strong broad-spectrum activity of these peptides [5], indicating their likely important role in the immune system of marsupials and monotremes. In the following sections, we will provide a brief overview of AMPs in mammals with a focus on the cathelicidin and defensin gene families, followed by a review of our current knowledge regarding these AMPs in marsupials and monotremes. We will also discuss the significance of studying AMPs in these divergent mammalian lineages.

## 2. Antimicrobial peptides (AMPs)

AMPs are a class of ancient host defence molecules that have been identified in every species studied thus far. They represent an important group of immunological proteins which provide chemical defence against bacteria, fungi, protozoa and viruses and are significant modulators of innate and adaptive immunity [13]. Characteristically, these peptides have a high proportion of positively charged residues which enable electrostatic interaction with negatively charged microbial lipid membranes [14]. Located predominantly within the mucous membranes, skin and phagocytic cells, AMPs have co-evolved over millennia with a variety of microorganisms and still remain effective defence molecules [14].

### 2.1. Mechanisms of antimicrobial activity

AMPs usually function by disrupting the negatively charged microbial membranes. Three models of membrane disruption have been proposed: the barrel stave model [15], the carpet pore model [16] and the toroidal pore (wormhole) model [17]. The barrel stave model involves the aggregation and perpendicular insertion of peptides, forming a barrel like ring within the target membrane [17, 18]. In this model electrostatic reactions facilitate insertion of the hydrophobic portions of the peptide and continual recruitment of peptides to result in a polydisperse pore [18]. The carpet model involves the binding of monomers to form a carpet like layer across the surface of the membrane, which disrupts the

 

curvature of the membrane and thereby leads to dissolution [18]. In the toroidal pore or wormhole model, pores are formed through lipid layer bending induced by AMP insertion and are stabilised by the AMPs [17, 18].

In addition to membrane permeabilisation, an increasing amount of evidence indicates that there are other complementary mechanisms involved in killing microbes by AMPs [19, 20]. Such mechanisms are associated with the interaction of AMPs with intracellular targets and include the inhibition of cellular functions such as the disruption of metabolic processes, protein and DNA [18]. Moreover, synergistic actions between AMPs are believed to be an essential factor required to exert their full effect [21]. Synergy has been observed between specific AMP families such as defensins and cathelicidins [19, 21], as well as defensins and other AMPs such as lactoferrin and lysozyme [22]. Such synergy between AMPs can result in augmented activity and increased potency towards microbial targets [21], which may explain why *in vivo* concentrations of naturally expressed AMPs are often found to be lower than their minimum inhibitory concentrations (MIC) deducted *in vitro* [19].

### 2.2. Co-evolution of AMPs and microbes

AMPs belong to the most rapidly evolving group of mammalian peptides [23]. The high evolution rate of AMPs can be largely attributed to the selective pressure exerted by ever-evolving pathogens. Microbes can develop various strategies to interfere with antimicrobial activity of the peptides and evade AMP-mediated host defence [19]. Down-regulation of AMP expression has been identified in certain bacteria to attenuate AMP activity [24]. Some microbes secrete proteases or polysaccharides with degradation or sequestering abilities [25]. Microbes can also manipulate their cell membrane composition to reduce negative charge and thereby decrease electrostatic attraction on which AMPs rely to target microbial membranes [26, 27].

As a result of the rapid evolution, while some AMPs remain conserved throughout various evolutionary lineages, numerous genes have undergone species/lineage-specific expansion or contraction via gene duplication and diversification events. To date, over 1200 AMPs have been identified in a variety of organisms ranging from microbes to animals [18, 19, 28], with defensins, cathelicidins, the insect cecropins and the amphibian magainins representing the most predominant AMP families [14]. Despite their similar capacities to directly kill or inhibit microbes, the immunological effect of specific AMP families is highly specialised and their expression variable between species [19].

### 2.3. Defensins

Defensins are small (3-5 kDa), highly basic and cationic AMPs that have been identified in a variety of life forms including plants, invertebrates and vertebrates [13, 29]. They have various roles in both innate and adaptive immunity, contributing to antimicrobial defence by directly inactivating microbial cells and acting as mediators of immunoregulatory processes such as the chemoattraction of host immune cells [13]. Their wide species distribution and broad spectrum of activity suggest they are key players in the host defence against a myriad of potential pathogens. Three defensin subfamilies have been described in vertebrates – alpha (α), beta (β) and theta (θ). Each subfamily is distinguished by a characteristically spaced cysteine residue framework within the mature peptide and the presence of conserved and distinct disulfide bridging patterns between connecting cysteine residues [30]. The disulfide bridging pattern in alpha defensins forms a 1-6, 2-4, 3-5 cysteine linkage and in beta defensins forms a 1-5, 2-4, 3-6 linkage [31]. Structurally, both alpha and beta defensins possess a characteristic triple stranded antiparallel β sheet scaffold that forms a distinct defensin fold and the structural core of the peptide [32-34]. Unlike alpha and beta defensins which are both open-ended peptides, theta defensins have a unique closed circular structure [35].

Alpha and beta defensins differ in their bacterial strain specificity, gram specificity and *in vitro* potency. A variety of characteristics account for these differences including primary structure, cationicity, hydrophobicity and three-dimensional structure [36]. The mature peptide sequence is highly variable between and within defensin subfamilies, which is believed to be a direct result of positive Darwinian selection favouring gene diversification [37]; nevertheless, many compositional and structural features remain conserved between alpha and beta defensins. These include 1) a high proportion of cationic amino acid residues, and 2) the six-cysteine-residue motif, with the second and fourth cysteines forming an intramolecular bonding, and the fifth and sixth cysteines adjacent to each other at the carboxyl terminal [37, 38].

The exact evolutionary relationship between defensin subfamilies remains uncertain, yet all three defensin subfamilies are believed to share a common evolutionary origin. Identified in both mammalian and avian species, beta defensins are believed to be the oldest family of vertebrate defensins [13, 39]. Recent studies have linked the emergence of beta defensins to an ancestral 'big defensin', which is distributed amongst athropoda, mollusca and cephalaochordata [40]. The evolutionary origin of the beta defensin domain is proposed to be traceable back to the common ancestor of bilateral metazoans [40]. Based on phylogenetic analysis, it is proposed that alpha defensins have arisen from relatively recent gene duplication events [41]. This is also supported by the fact that alpha defensins have only been identified in mammalian lineages. Theta defensins are the most recently discovered defensin subfamily and to date their presence remains exclusive to the old world primate lineages [31]. Initially identified in the leukocytes of the rhesus macaque (*Macaca mulatta*), theta defensins were the first known circular peptides in mammals and likely have originated from

the mutation of an alpha gene [42]. Theta defensin-like loci have been identified in humans, but are non-functional due to a mutation resulting in an in-frame stop codon [43].

Like other AMPs, defensins exert their function mainly by pore forming mechanisms. Charge and hydrophobicity are two key molecular characteristics of defensins that are associated with selective targeting and interaction with eukaryotic and prokaryotic cells. For previously reported defensins, the charge of mature peptides ranges between +1 to +11, attributed to the high proportion of positively charged arginine and lysine residues [44]. The expression and secretion of defensins is regulated by a variety of factors and is dependent on the cell type, microenvironment and stimuli [45]. It can also vary greatly between species, which has been related to evolutionary pressures from species-specific pathogens [31]. Generally, defensins are predominantly expressed in host-defence-associated cells, such as neutrophils, macrophages, monocytes and keratinocytes [46]. In addition, they have been isolated from a range of tissues, predominantly the mucosal epithelial cells of the digestive, respiratory, urinary and reproductive systems [19, 47, 48]. The expression of defensins can be constitutive and/or induced by microbial antigens and pro-inflammatory stimuli [30, 36, 37, 49, 50].

### 2.4. Cathelicidins

First discovered in the cecropia moth (*Hyalophora cecropia*) in 1980 [51], cathelicidins are a family of ancient AMPs which pre-dates the evolution of vertebrates [52, 53]. Unlike defensins which usually exist in high copy numbers in mammals, the number of cathelicidins is relatively low in many characterised species, such as the human, mouse and rat which only have one copy of the gene. Cathelicidin genes encode a propeptide consisting of a cathelin domain, which is conserved across species, and a highly variable antimicrobial domain, which forms the active mature peptide. The size of cathelicidins in different species varies greatly from 12 to 100 amino acid residues, but one common feature is the high percentage of arginine or lysine residues within the sequence conferring a high cationic charge for antimicrobial activity [54]. Similar to defensins, cathelicidins are constitutively expressed in epithelial cells of the respiratory, gastrointestinal and reproductive systems, whereas in certain cell types, such as keratinocytes, their expression are induced by inflammatory stimuli [55, 56]. Neutrophils are the major site of cathelicidin secretion within circulation [57]. Agranular leukocytes such as T and B lymphocytes, natural killer cells and macrophages also contribute to the circulating level of cathelicidins [58].

In addition to direct antimicrobial activity, cathelicidins play significant roles in immune modulation. For example, bovine, human, mouse and porcine cathelicidins are suggested to be chemotactic to almost all populations of blood cells [59]. Neutrophil degranulation and release of cathelicidins can form a chemotactic gradient which induces the directional migration of effector cells in a dose dependent manner. Cathelicidins can also modulate immune responses by inducing the release of pro-inflammatory molecules such as histamine and interleukins from a variety of cells [59-61]. Stimulation of mast cells by LL-37, the human cathelicidin, causes degranulation and release of histamine, which increases vascular permeability and thereby enables entry of immune cells to the site of infection [61, 62]. Similarly, cathelicidins can induce the release of cytokines interleukin-8 [63] and interleukin-1-beta [60] to produce intense and sustained inflammatory responses.

## 3. Defensins and cathelicidins in marsupials and monotremes

AMP gene discovery and characterisation has been carried out in three marsupial or monotreme species, the grey short-tailed opossum [9], tammar wallaby [10] and platypus [11, 12], whose genome has been fully sequenced. Numbers of identified defensins and cathelicidins are summarised in Table 1 and 2, respectively. Although the study of marsupial and monotreme AMPs is still in a preliminary stage, some important findings have been made, revealing the likely crucial role of AMPs in these species.

**Table 1** Summary of defensins identified in marsupial and monotreme species

| Species | Number of alpha defensins | Number of beta defensins | Reference |
|---|---|---|---|
| Platypus | 4 | 6 | [12] |
| Opossum | 1 | 32 | [9] |

 

**Table 2** Summary of cathelicidins identified in marsupial and monotreme species

| Species | Gene name | Peptide name | Reference |
|---|---|---|---|
| Tammar wallaby | *MeauCath1* | CATH1 | [10] |
| | *MeauCath2* | CATH2 | |
| | *MeauCath3* | CATH3 | |
| | *MeauCath4* | CATH4 | |
| | *MeauCath5* | CATH5 | |
| | *MeauCath6* | CATH6 | |
| | *MeauCath7* | CATH7 | |
| | *MeauCath8* | CATH8 | |
| Grey short-tailed opossum* | *MdoCATH1* | CATH1 | [9] |
| | *MdoCATH2* | CATH2 | |
| | *MdoCATH3* | CATH3 | |
| | *MdoCATH4* | CATH4 | |
| | *MdoCATH5* | CATH5 | |
| | *MdoCATH6* | CATH6 | |
| | *MdoCATH7* | CATH7 | |
| | *MdoCATH8* | CATH8 | |
| | *MdoCATH9* | CATH9 | |
| | *MdoCATH10* | CATH10 | |
| | *MdoCATH11* | CATH11 | |
| | *MdoCATH12* | CATH12 | |
| Platypus* | *Cath1* | CATH1 | [11] |
| | *Cath2* | CATH2 | |
| | *Cath3* | CATH3 | |
| | *Cath4* | CATH4 | |
| | *Cath5* | CATH5 | |
| | *Cath6* | CATH6 | |
| | *Cath7* | CATH7 | |
| | *Cath8* | CATH8 | |

* The cathelicidin sequences in these species were deduced from genomic data and have not been experimentally confirmed.

### 3.1. Lineage-specific gene expansion

Marsupial and monotreme AMPs are found to have undergone linage-specific gene expansion, giving rise to high gene copy number and diversity [9, 64]. For instance, in the grey short-tailed opossum, as many as 32 beta defensins have been identified (Table 1); moreover, twelve cathelicidins have been annotated in the same species (Table 2), which is the largest number of all species studied. These cathelicidins of the opossum are highly variable, with the sequence similarity ranging between 5-95%. Phylogenetic analyses demonstrated that most marsupial and monotreme AMP sequences form lineage or species-specific clades that are diverse from eutherian AMPs [9, 10], suggesting that the multiple copies of AMPs are likely attributed to recent gene duplication events after the lineage/species divergence

followed by rapid sequence diversification. The expansion or contraction of immune gene families in a species is largely driven by the selective pressure caused by pathogenic challenges the species encounter [37]. Therefore, the unique reproductive characteristics of marsupials and monotremes are likely to have encouraged amplification and diversification of defensin and cathelicidin gene families in these lineages, as possessing a large number of AMPs with diverse specificities may help the immunological naïve neonates fight off a wide range of microbes in the pouch or the burrow.

### 3.2. Broad-spectrum and highly potent antimicrobial activity

Different AMPs kill bacteria, fungi and parasites with differing levels of efficiency [54]. By synthesising three tammar wallaby and two platypus cathelicidins *in vitro* and testing for activity, Wang and colleagues [5] demonstrated that these marsupial and monotreme AMPs have effective antimicrobial activity against a wide range of pathogens, including gram negative and positive bacteria as well as fungi. One wallaby cathelicidin, WAM1, shows potent activity up to 80 times higher than the human cathelicidin LL-37 against certain microbes, and is over ten times more effective than antibiotics such as ampicillin, tetracycline and chloramphenicol against *E. coli* and *B. subtilis*. Moreover, this marsupial AMP shows capacity to kill multi-drug resistant clinical bacterial isolates including *P. aeruginosa*, *A. baumannii* and *K. pneumonia*. The evolution of such high potency of marsupial and monotreme AMPs is likely to have been driven by the need to remove pathogens in the pouch and maintain the health of their altricial young.

### 3.3. Protection in the pouch

The exact role of defensins and cathelicidins in neonatal protection in marsupials and monotremes is not fully understood with very limited studies conducted so far. But based on knowledge from other well-studied species, we propose that these AMPs may perform a crucial protective role in several different ways.

#### 3.3.1. *Protection by the mother*

##### Milk

It is well known that human milk contains significant amounts of the antibacterial compounds lysozyme and transferrin. Meanwhile, high concentrations of defensins HBD-2, HBD-1, HD-5, HD-6 and HNP-1 are also detected [65], with the expression level greater than that in other mucosal surfaces [66]. Cathelicidin LL-37 and defensin HBD-1 mRNA has been found to be expressed by immune cells in milk such as macrophages, lymphocytes and neutrophils [65]. The hormonal milieu of pregnancy is believed to induce elevated expression of defensins, hence the expression level can change over the course of lactation [66, 67] Links have been drawn between the antimicrobial compounds in milk and the prevalence of common newborn conditions [65]. Human cathelicidin LL-37 is active against *E.coli* and *L.monocytogenes*, which are common causes of neonatal sepsis. Furthermore, formula-fed newborns are more likely to develop respiratory infections, diarrhoea and allergies [65].

Similar to humans, AMPs are secreted into milk throughout the whole span of lactation in tammar wallabies [68]. Studies failed to detect cathelicidins in platypus milk, but this could be an artefact caused by sampling difficulty and requires further exploration [8]. In tammar wallabies, release of colostrum coincides with an increased cathelicidin (MeauCath1) expression, which continues to rise throughout the second phase of immunoglobulin transfer around 100 days post-partum [3, 68]. This signifies their protective function over the course of pouch life, especially prior to maturation of humoral immunity.

##### Reproductive tract

Beta defensins and cathelicidins have been identified in the female reproductive tract of a variety of species and are recognised as a key defence mechanism against ascending infection [42]. In humans, expression varies throughout the reproductive tract, with high levels localised to the cervix and vagina and thereby preventing infection [52]. Human beta defensins have also been isolated from the epithelial cells of the placenta and may play a role in protection of the maternal/foetal interface [69]. The expression of beta defensins is affected by sex hormone levels [42]. The presence of progesterone upregulates the epithelial cell expression of beta defensin in the fallopian tubes of sheep in a concentration dependent manner [42, 70].

Human cathelicidin LL-37 has been detected at functional levels in the vernix caseosa, a white substance that covers the newborn at birth and acts as a natural biofilm in-utero [71, 72]. LL-37 is secreted by the foetus in-utero and has activity against gram positive bacteria [72]. The egg coating in reptile and avian species plays a similar role to the vernix caseosa in humans. Beta defensins have been identified in chicken uterine epithelial cells and the oviduct with high levels of expression in the infundibulum and vagina [73]. They have also been isolated from the surface fibres and protein matrix of the egg. Therefore, different from the vernix caseosa, this suggests maternal secretion of AMPs which coat the egg and contribute to the defence of the developing embryo. Monotremes are egg-laying mammals with

 

reproductive tract more closely resembles that of avian species. Given this similarities, we speculate that the coating on monotreme eggs may contain AMPs of maternal origin, which is worth further investigation.

Pouch

The protein profile of the tammar wallaby pouch varies significantly over the reproductive life of the female, with the greatest diversity of peptides isolated from the mature active pouch [74]. This fluctuation has been attributed to increased secretion, originating from either the maternal pouch tissue or from young itself. AMPs are secreted in the post-reproductive pouch of the tammar wallaby [74], which contribute to changes in pouch flora by selectively eliminating potential pathogens [10]. In both the tammar wallaby and quokka, a decrease in gram negative bacteria was observed leading up to parturition. At the time of birth, levels of gram negative bacteria were virtually zero and remained very low for up to 6 weeks [75, 76]. A decrease in gram positive bacteria such as *Staphylococcus aureus* has also been observed in possums with pouch young [77]. These observations strongly suggest that the secretion of AMPs in the pouch may play a significant part in young protection.

3.3.2. *Self-defence of the young*

Immune cells

As mentioned previously, leukocytes in the opossum young proliferate rapidly during the first two weeks after birth with neutrophils being the predominant cell type [7, 8]. Neutrophils are a predominant site of cathelicidin and alpha defensin expression. In humans, alpha defensins constitute 5-7% of total cellular protein and 30-50% of the protein contents of azurophil granules [33]. Therefore, the large increase in leukocytes during opossum neonatal development may be contributing to high circulating levels of AMPs within the young.

Skin

In mice, embryonic and newborn mice express cathelicidin CRAMP at higher levels in skin when compared to adults [78]. Human cathelicidin and beta defensins are constitutively expressed in the epidermis of newborns, whereas expression in adults is induced by inflammatory stimuli [72, 79]. Such increased expression in newborn skin indicates that AMPs play a critical role in primary defence of the neonate at the epithelial barrier [71].

Respiratory tract

Human beta-defensin 2 and cathelicidin have both been detected at functional levels in the airway mucosal surface of newborns, directly interacting with pathogens in the respiratory tract and protecting the newborns from pulmonary infection [80]. Similarly, it has been found that the expression of tammar wallaby cathelicidin MeauCath7 in the lung rises over the first 25 days post-parturition as the young encounters air-borne pathogens [81].

Gastrointestinal tract

In adults, defence of intestinal mucosa is boosted by the secretion of cathelicidins from paneth cells within the epithelium. Although these cells are not fully developed until after the neonatal period, neonatal mice show constitutive expression of active CRAMP in intestinal epithelial cells [82]. Thus, a mechanism exists in neonates to circumvent the absence of paneth cells and produce functional AMPs to overcome the increased contact with pathogens after leaving the uterus [82]. In the tammar wallaby, the cathelicidin MeauCath8 is expressed in the GIT of neonates from day 1 after birth [81]. In addition, the expression of MeauCath7 is increased in the jejunum, liver and spleen for the first 25 days of life [81]. As such, AMPs may also play a role in neonate intestinal defence in marsupials and monotremes.

## 4. Conclusions and future prospects

Due to their unique reproductive physiology, marsupials and monotremes are born highly under-developed and have to face a wide range of pathogenic challenges in the pouch or burrow from an early developmental stage. One important strategy these animals have evolved to deal with such challenges is to produce a large and diverse collection of antimicrobial peptides, which have broad-spectrum activity against microbes and can be significantly more potent than eutherian peptides. These AMPs are likely to represent a vital line of protection for the immunologically naïve young prior to immune system maturation.

Marsupials and monotremes occupy a unique evolutionary position between reptiles and eutherian mammals. Further studies are needed to characterise more AMPs in these animal lineages. This not only will contribute to our knowledge of the immune system in the species, but will also improve our understanding on the history and mechanism of the evolution of immunity in the animalia kingdom.

Moreover, the dwindling number of therapeutic options for emerging bacterial 'superbugs' means that development of novel antibiotics is of utmost importance. Given the large gene copy number and high sequence diversity, marsupial and monotreme AMPs provide a promising source for developing new antimicrobials with potent and broad-spectrum activity to overcome the problem of drug resistant microbes.

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