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Review

## Avian defensins

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### Abstract

Modulation of defensin expression may be one way to improve animal health and to reduce zoonotic diseases. Defensins are small, cationic, and amphipathic cysteine-rich antibiotic peptides found in plants, insects, mammals and birds. Whereas  $\alpha$ - and  $\theta$ -defensins appear to be absent in birds, several  $\beta$ -defensins have been isolated from avian heterophils. In addition,  $\beta$ -defensins were found to be constitutively or inducibly expressed at mucosal surfaces of the respiratory, intestinal and urogenital tracts. In this review the current knowledge of the defensin repertoire of birds, their tissue-specific expression, regulation and corresponding biological functions are described.

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**Keywords:** Defensins; Antimicrobial Peptides; Innate immunity; Birds

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**Abbreviations:** aa, amino acid; AMP, antimicrobial peptide; AvBD, avian  $\beta$ -defensin; ATCC, American type culture collection; CHP, chicken heterophil peptide; Def, defensin; ER, endoplasmic reticulum; Gal, gallinacin; GPV-1, gallopavin-1; hBD, human  $\beta$ -defensin; IGF-1, insulin growth factor 1; MRSA, methicillin-resistant *Staphylococcus aureus*; Osp, ostricacin; RT-PCR, reverse transcriptase polymerase chain reaction; SCCE, stratum corneum chymotrypsin-like enzyme; SCTE, stratum corneum trypsin-like enzyme; SNP, single nucleotide polymorphism; Sphe, Sphenicin; TGF-1, transforming growth factor 1; THP, turkey heterophil peptide; UTR, untranslated region.

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## 1. Introduction

Birds are important reservoirs of zoonotic pathogens. Human pathogens, such as *Salmonella* and *Campylobacter* spp., may reside in the chicken intestinal tract without giving rise to clinical symptoms, but are major causes of food poisoning (Helms et al., 2006; Kessel et al., 2001). Domesticated birds are also an important reservoir of avian influenza A virus subtypes that pose a threat to both animal and human health (e.g. H5N1) and have been linked to regularly reoccurring outbreaks of influenza in Asia (Kung et al., 2007). Thus, animal health and public health will greatly benefit if the innate immune system of domesticated birds can be boosted to limit or even prevent colonization and spreading of zoonotic pathogens.

Prime targets for boosting of innate immunity are defensins, a family of small cationic peptides with broad spectrum antimicrobial activity against bacteria, fungi, protozoa and enveloped viruses (Zasloff, 2002). Defensins, a subset of antimicrobial peptides, are cystine-rich peptides that vary in length from 18 to approximately 45 amino acids and are enriched in hydrophobic and cationic aa residues (Selsted and Ouellette, 2005). In addition to their direct antimicrobial activities, immunomodulatory properties have also been demonstrated. Defensins can promote adaptive immunity by selective recruitment by chemotaxis of monocytes (Territo et al., 1989), T lymphocytes (Chertov et al., 1996), immature dendritic cells (Yang et al., 1999) and mast cells (Niyonsaba et al., 2002) to sites of inflammation. Furthermore, they are able to induce histamine release from peritoneal mast cells (Befus et al., 1999) and to enhance macrophage phagocytosis (Fleischmann et al., 1985; Ichinose et al., 1996).

Anti-inflammatory properties have also been attributed to defensins, such as inhibition of formylpeptide receptor-mediated chemotaxis of polymorphonuclear leukocytes (Grutkoski et al., 2003) and the binding of bacterial endotoxins (Motzkus et al., 2006). In addition, defensins may enhance wound repair by inducing fibroblast (Murphy et al., 1993) and epithelial cell proliferation (Aarbiou et al., 2002, 2004; Murphy et al., 1993). In vertebrates, three different defensin subfamilies ( $\alpha$ ,  $\beta$  and  $\theta$ ) exist, differing in disulfide bridge

pairing and positioning of their conserved six cysteine residues, Cys1–Cys6, Cys2–Cys4, Cys3–Cys5 for  $\alpha$ -defensins versus Cys1–Cys5, Cys2–Cys4, Cys3–Cys6 for  $\beta$ -defensins (Selsted and Ouellette, 2005). The observation that  $\alpha$ - or  $\theta$ -defensins have not been found in phylogenetically much older vertebrates, such as birds and fish, suggests that all defensin subfamilies must have evolved from an ancestral  $\beta$ -defensin gene by duplication and diversification (Semple et al., 2003).

Several  $\beta$ -defensins have been described for avian species, in particular for galliformes, either isolated from heterophilic granulocytes or discovered by *in silico* analysis (Table 1). In the following sections the current status of knowledge regarding avian defensins is described, with the emphasis on localization, putative functions, biosynthesis and regulation, and where necessary, the relation to their mammalian counterparts. For practical purposes, the nomenclature of chicken  $\beta$ -defensin and related sequences from other avian species in this review is based on the recently proposed update of the avian  $\beta$ -defensin nomenclature by Lynn et al. (2007).

## 2. Genomic organization

At least 14 chicken  $\beta$ -defensin genes are located in a ~86.0 kb single  $\beta$ -defensin cluster on chromosome 3q3.5–q3.7 (Lynn et al., 2007; Xiao et al., 2004). Chicken  $\beta$ -defensin genes consist of four exons (Fig. 1), with the exception of the AvBD12 gene where the last two exons have fused (Xiao et al., 2004). Defensins are synthesised as inactive precursors, i.e. prepropeptides, consisting of a short signal peptide, a propiece and the mature peptide. The propiece is often but not always anionic. For example, AvBD1 and -2 do not contain a negatively charged propiece (Brockus et al., 1998). The 1st exon corresponds to the 5'UTR region, the 2nd exon encodes the signal peptide and part of the propiece, while the remaining part of the short propiece and majority of the mature peptide are encoded by the 3rd exon. The remaining part of the mature peptide and the 3'UTR region are encoded by the 4th exon (Fig. 1). The chicken 3q3.5–q3.7  $\beta$ -defensin locus appears to have evolved by a series of gene duplications, followed by substantial divergence of the exon(s) encoding the mature peptide with substantial “positive selection”,

Table 1  
Nomenclature of avian defensins

Designation	Synonyms	Genbank	References
<b>Chicken</b>			
AvBD1	Gal-1/1 $\alpha$ ; CHP-1	AAB30584	Evans et al. (1994) and Harwig et al. (1994)
AvBD2	Gal-2	AAB30585	Harwig et al. (1994)
AvBD3	Gal-3	Q9DG58	Zhao et al. (2001)
AvBD4	Gal-7; Gal-4	AAS99318	Lynn et al. (2004) and Xiao et al. (2004)
AvBD5	Gal-9; Gal-5	AAS99320	Lynn et al. (2004) and Xiao et al. (2004)
AvBD6	Gal-4; Gal-6	AAS99315	Lynn et al. (2004) and Xiao et al. (2004)
AvBD7	Gal-5; Gal7	AAS99316	Lynn et al. (2004) and Xiao et al. (2004)
AvBD8	Gal-12; Gal-8	AAU07922	Higgs et al. (2005) and Xiao et al. (2004)
AvBD9	Gal-6; Gal-9	AAS99317	Lynn et al. (2004) and Xiao et al. (2004)
AvBD10	Gal-8; Gal-10	AAS99319	Lynn et al. (2004) and Xiao et al. (2004)
AvBD11	Gal-11	AAT45551	Xiao et al. (2004)
AvBD12	Gal-10; Gal-12	AAS99321	Lynn et al. (2004) and Xiao et al. (2004)
AvBD13	Gal-11; Gal-13	AAT48937	Higgs et al. (2005) and Xiao et al. (2004)
AvBD14	Gal-14	AM402954	Lynn et al. (2007)
<b>Turkey</b>			
AvBD1	THP-1	AAC36053	Evans et al. (1994)
AvBD2	THP-2	AAC36054	Evans et al. (1994)
AvBD3	GPV-1	AAG09213	Zhao et al. (2001)
<b>Mallard duck</b>			
AvBD2	Duck $\beta$ -def.	AAV52799	Lynn et al. (2007)
AvBD9	Duck $\beta$ -def.-6-like	ABN50328	Lynn et al. (2007)
<b>King pigeon</b>			
AvBD4	King pigeon $\beta$ -def.	ABI20694	Lynn et al. (2007)
<b>Ostrich</b>			
AvBD1	Osp-2	P85114	Sugiarto and Yu (2006)
AvBD2	Osp-1	P85113	Yu et al. (2001)
AvBD4	Ostrich gallinacin-4	ABK40533	Lynn et al. (2007)
AvBD7	Osp-3	P85115	Sugiarto and Yu (2006)
AvBD8	Osp-4	P85116	Sugiarto and Yu (2006)
<b>King penguin</b>			
AvBD103a	Sphe-1	P83429	Thouzeau et al. (2003)
AvBD103b	Sphe-2	P83430	Thouzeau et al. (2003)

i.e. involving mutations that disproportionately favour the selection of charged aa residues. This is further emphasized by the finding of phylogenetically more conserved genes, such as cathepsin B and HARL2754, in close proximity of chicken, mouse and human  $\beta$ -defensin clusters (Xiao et al., 2004) and suggests that vertebrate  $\beta$ -defensins originate from a single ancestral gene.

Single-nucleotide polymorphisms (SNPs) are commonly found in  $\beta$ -defensin genes, and although present only at a low frequency in the coding region, the occurrence of SNPs may seriously affect an individual's predisposition to disease (Braida et al., 2004), by altering the efficiency of transcription and translation or by generating an altered protein sequence with diminished biological functions (Jurevic et al., 2002). SNP analysis of the chicken  $\beta$ -defensin cluster revealed

a much higher SNP rate (13.2 SNPs/kb) for the 3.25 kb region containing the AvBD2, -3, -4, -5 and -7 genes, than the rate across the whole chicken genome (5 SNPs/kb) as was previously reported (Wong et al., 2004). However, all 43 identified SNPs were intronic, with the exception of a nonsynonymous SNP found in the AvBD5 gene that resulted in an amino acid substitution of proline to threonine. In addition to the variability in gene nucleotide sequence, studies in humans have shown that specific defensin genes may be completely absent in some individuals (Ballana et al., 2007). Moreover, in contrast to other innate immune genes, defensins may show a high degree of polymorphism in gene copy number (Hollox et al., 2003; Linzmeier and Ganz, 2005, 2006), the latter of which has been linked to predisposition to Crohn's disease of the colon in humans (Fellermann et al., 2006). Few studies have

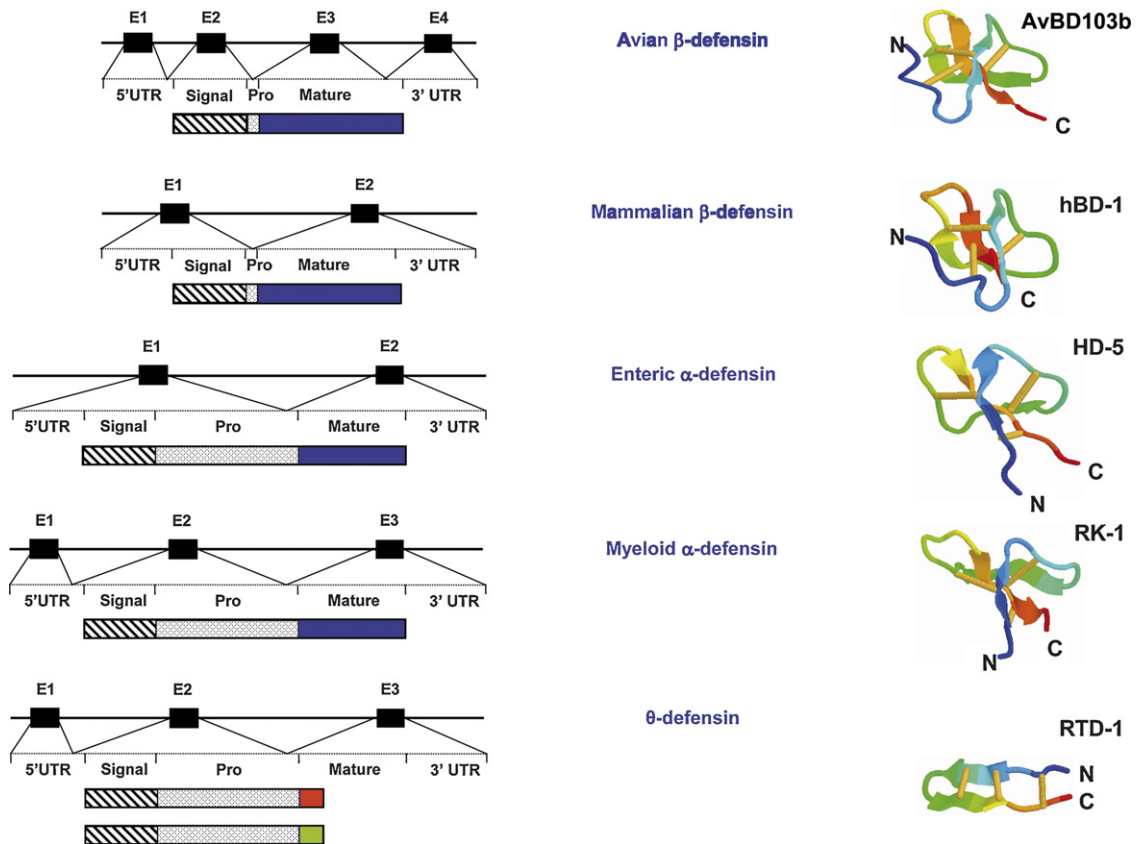


Fig. 1. Genomic organization of avian and mammalian defensins and corresponding mature peptide structures. Differential transcription and subsequent translation of exons (E1–4) into the 5' and 3' untranslated regions (UTR) and peptide encoding regions for: signal peptide (diagonal striped bars), propeptide (dotted bars) and mature peptide (solid blue bars; red and green bars depict homo- or heterodimers based formation of a single cyclic  $\theta$ -defensin peptide). Despite differences in genomic organization, 3D structures of mature avian  $\beta$ -defensins and mammalian  $\alpha$ - and  $\beta$ -defensins are very similar: AvBD103b, king penguin avian  $\beta$ -defensin 103b (Spheniscin-2); hBD-1, human  $\beta$ -defensin-1; HD-5, human  $\alpha$ -defensin-5; RK-1, rabbit kidney defensin-1; RTD-1, rhesus  $\theta$ -defensin-1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

addressed the disease predisposition of birds in relation to innate immune gene polymorphisms. Hasenstein et al. (2006) addressed the association of avian  $\beta$ -defensin gene polymorphisms in 1-week-old  $F_1$  chickens with their phenotypic immune response to *S. enteritidis* challenge. In this study, AvBD2 and -5 gene polymorphisms were moderately associated with caecal and spleen bacterial loads at 1 week post-challenge. The significant associations observed between polymorphisms of the AvBD3 and AvBD7 genes and *S. enteritidis* vaccine antibody response at 21 days, suggest that avian  $\beta$ -defensin genes may facilitate the transition from an innate immune response to an adaptive immune response in newly hatched birds (Hasenstein et al., 2006). Thus, polymorphisms in avian innate immune genes, encoding signalling pathway components as well as innate immune effectors, can be directly associated with disease resistance. Hence, the allelic selection of

genes involved in host immunity by selective breeding may confer an increased efficiency of the innate immune system (Georges, 2001). However, it remains to be elucidated to which extent gene polymorphisms (SNPs) and variations in  $\beta$ -defensin copy number play a role in the resistance of birds to *Salmonella* infections.

### 3. Structural features

Deduced primary amino acid sequences for avian mature defensins indicate that, similar to mammalian  $\beta$ -defensins (Pazgier et al., 2006), these peptides consist of 36 aa or more residues with the consensus sequence motif:  $x_n$ -C-x<sub>2-4</sub>-G-x<sub>1-2</sub>-C-x<sub>3-5</sub>-C-x<sub>9-10</sub>-C-x<sub>5-6</sub>-CC-x<sub>n</sub>. NMR spectroscopy analysis of synthetic king penguin AvBD103b in aqueous solution revealed a three-dimensional structure most similar to mammalian  $\beta$ -defensins (Landon et al., 2004) (Fig. 1). The overall

fold consisted of a three-stranded  $\beta$ -sheet and an  $\alpha$ -helical N-terminus in the structure and contained a hydrophobic patch (Phe<sup>19</sup>-Pro<sup>20</sup>-Ile<sup>22</sup>-Val<sup>37</sup>-Trp<sup>38</sup>), shown by comparative structure analysis to be well but not strictly conserved in other avian defensins. The 10 Arg residues and lack of Glu or Asp residues renders king penguin AvBD103b highly cationic (10+). Some avian  $\beta$ -defensin genes, e.g. AvBD3, -11 and -13, contain a large postpiece, although the AvBD13 nucleotide sequence reported by Higgs et al. lacks this postpiece suggesting that there might be strain-specific splice variants or isoforms of the AvBD13 gene (Higgs et al., 2005). Comparative analysis of the nucleotide sequences of chicken and turkey AvBD3 (91% identical on a nucleotide level), the latter of which lacks a postpiece, revealed that a two-base insertion just before the chicken AvBD3 original stop codon, causes a frame shift, and an additional 15 bp insertion was responsible for the generation of an anionic postpiece in chicken AvBD3 (Zhao et al., 2001). The AvBD11 postpiece contains a defensin-like motif: x<sub>9</sub>-C-x<sub>4</sub>-G-x-C-x<sub>6</sub>-C-x<sub>7</sub>-C-x<sub>6</sub>-CC-x<sub>3</sub>, which might be a consequence of gene duplication. Interestingly, of the newly identified human  $\beta$ -defensins mapped to chromosomes 6 and 20 (hBD-18 to 21, hBD-23, hBD-25 to 29 and hBD-31), those clustered on chromosome 20 all contained similar long C-terminal tails (Schutte et al., 2002). These C-terminal tails differ substantially in amino acid composition and do not exhibit homology with other sequences (Pazgier et al., 2006). It has been suggested that the accumulated negative charges present in some of these large postpieces could function similarly to the anionic charges present in the  $\alpha$ -defensin propiece (Rodríguez-Jiménez et al., 2003). The net anionic charges in the propiece of  $\alpha$ -defensins are thought to balance out the cationic net charge of the mature peptide, which might be important for folding and prevention of intracellular binding to membranes (Liu and Ganz, 1995; Michaelson et al., 1992; Valore et al., 1996; Wu et al., 2007). The biological function of these large postpieces and their putative role during folding and intracellular trafficking remains to be elucidated.

#### 4. Biosynthesis and tissue-specific processing

Although mechanisms ruling the synthesis, storage and activation of avian  $\beta$ -defensins are unknown, they can be expected to parallel that of mammalian defensins. Avian  $\beta$ -defensins have been isolated from heterophils of chickens, turkeys and ostrich (Evans et al., 1995; Harwig et al., 1994; Sugiarto and Yu, 2006)

or were found to be constitutively or inducibly expressed by epithelial cells (Zhao et al., 2001).

Myeloid  $\beta$ -defensins are synthesized in the bone marrow, where in the Golgi apparatus and maturing granules of promyelocytes the prodefensins are processed by as yet unknown proteases and stored into specific granules as mature peptides (Yount et al., 1999). The abundant mRNA levels of AvBD4 to -7 found in chicken bone marrow, and the absence of significant AvBD4 to -7 expression in leukocyte extracts (Xiao et al., 2004), suggest that, similar to mammalian defensins (Landon et al., 2004; Yount et al., 1999), myeloid avian  $\beta$ -defensin mRNA synthesis is largely absent in mature leukocytes. After biosynthesis and intracellular trafficking through the Golgi apparatus, the  $\sim$ 19 aa signal peptide, that functions to anchor the prodefensin peptide in the endoplasmic reticulum (ER) membrane, is rapidly proteolytically cleaved to generate a prodefensin peptide with little or no microbicidal activity (Satchell et al., 2003). The large ( $\sim$ 40 aa)  $\alpha$ -defensin propiece has demonstrated to be essential for subcellular trafficking and sorting of pro- $\alpha$ -defensins into specific secretory granules of polymorphonuclear leukocytes (neutrophils) (Liu and Ganz, 1995). The fact that  $\beta$ -defensins possess only a relative small propiece suggests that the subcellular trafficking of pro- $\beta$ -defensins may be different. Non-myeloid biosynthesis of  $\beta$ -defensin has been described to some detail for mammalian epithelial cells (Dale et al., 2001; Oren et al., 2003). In birds, non-myeloid  $\beta$ -defensin expression has been found for epithelial cells (Ohashi et al., 2005; Yoshimura et al., 2006; Zhao et al., 2001), but data describing their biosynthesis in these cells and tissues has not yet been reported. Paneth cells (known to contain large amounts of  $\alpha$ -defensins) are specialized secretory cells located at the base of the crypts of Lieberkühn in some species. Paneth cells or related cells were reported to be absent in ostrich, but have not been investigated in other avian species (Porter et al., 2002). It remains to be examined if birds possess Paneth-like cells that contribute to intestinal innate immunity.

The proteolytic processing of  $\beta$ -defensin proforms is host and tissue-specific due to the local repertoire of proteolytic enzymes and inhibitors and may result in multiple forms with different properties. For instance, human epididymis  $\beta$ -defensin-like peptides (HE2, ESP13.2, Bin1b, E-2, EP2, HE2) are processed by furin-like convertases, major processing enzymes of the secretory pathway located in the trans-Golgi network (Gu et al., 2001; von Horsten et al., 2002). hBD-1 produced by oral keratinocytes is processed into one major form (47 aa) and several minor forms (40–44 aa)

(Diamond et al., 2001) by yet unidentified proteases, whereas multiple truncated forms of hBD-1 occur in kidney and plasma, the latter of which could be explained by prodefensin cleavage by a chymotrypsin-like enzyme (Hiratsuka et al., 2000; Zucht et al., 1998).

Furthermore, details of  $\beta$ -defensin intracellular trafficking, storage and activation in myeloid and non-myeloid cells in general, and for avian defensins in particular, are lacking.

## 5. Tissue-specific gene expression

Beta-defensin-like sequences have been described for several domestic and wild bird species, of which the repertoire and putative functions have been most extensively investigated in domestic chicken.

### 5.1. Cell populations

Chicken  $\beta$ -defensins AvBD1 and -2, originally isolated from peripheral leukocytes (Harwig et al., 1994), and AvBD4–7, are all strongly expressed in bone marrow, whereas weak or no mRNA expression was found for AvBD4–7 in heterophils (Table 2). Together with the reported isolation of multiple  $\beta$ -defensins from turkey and ostrich heterophils (Evans et al., 1994; Sugiarto and Yu, 2006; Yu et al., 2001), this shows that avian heterophils, like the neutrophils of some mammalian species (Schneider et al., 2005; Selsted et al., 1993), contain multiple  $\beta$ -defensins.

### 5.2. Respiratory tract

In the respiratory tract, high  $\beta$ -defensin expression is observed for AvBD3 (Zhao et al., 2001) and AvBD9 (van Dijk et al., 2007) in trachea. In lung tissue moderate to strong expression is found for AvBD1 and -2 (Lynn et al., 2004; Zhao et al., 2001). Most other  $\beta$ -defensins are weakly or moderately expressed in these tissues (Table 2). Air sac membranes have not been extensively examined for  $\beta$ -defensin presence, but AvBD3 and -13 expression has been detected (Higgs et al., 2005; Zhao et al., 2001).

### 5.3. Skin

Chicken skin was shown to express moderate levels of AvBD3, -9 and -11 (van Dijk et al., 2007; Xiao et al., 2004; Zhao et al., 2001). Another avian  $\beta$ -defensin, designated AvBD14, has been recently deposited in the Genbank database (AM402954) and has been observed to be predominantly expressed in chicken skin (Dr. Pete

Kaiser, personal communication). The expression levels of  $\beta$ -defensins in chicken skin agrees with the reported basal expression of hBD-1–4 in keratinocytes of normal human skin, of which the latter three were strongly increased during induced keratinocyte differentiation (Harder et al., 2004). hBD-2 is a major constituent of psoriatic skin, a chronic non-infectious disease in which surprisingly few cutaneous infections occur (Harder and Schröder, 2005), whereas hBD-2 and other AMPs are deficient in atopic dermatitis, in which bacterial and viral skin infections are a recurrent problem (Ong et al., 2002). Thus, considering their presence in chicken skin, avian  $\beta$ -defensins may contribute to skin innate immune defense in birds.

### 5.4. Digestive tract

With the exception of AvBD11, weak to strong mRNA expression of chicken  $\beta$ -defensins is found throughout the digestive tract. In the proximal digestive tract, strong expression is observed for AvBD3 and -5 (Lynn et al., 2004) in tongue and AvBD9 (van Dijk et al., 2007) in esophagus and crop tissue. The crop is an extension of the esophagus in which food can be stored for up to 24 h and is well developed in gallinaceous birds. As chickens practice coprophagy to recover vitamins, amino acids and other nutrients produced by their hindgut bacteria (Montrose et al., 1985), an adequate local innate immune system is required. The high expression levels of AvBD9 in adult chicken crop tissue and its variable expression in juvenile broilers indicate an important role of AvBD9 in crop tissue defense (van Dijk et al., 2007).

In the glandular and muscular stomach,  $\beta$ -defensins are practically absent, apart from moderate AvBD9 (van Dijk et al., 2007) and AvBD13 (Higgs et al., 2005) expression levels found in the proventriculus. In the intestinal tract of newly hatched chickens, AvBD1 and AvBD2 mRNA levels were found to decrease during the first week and increase during the second week post-hatch (Bar-Shira and Friedman, 2006). Likewise, developmental expression studies of chicken AvBD4 mRNA using 1-, 4-, 17- and 38-day-old animals showed AvBD4 expression to be maximal within the first week post-hatch and to decline thereafter (Milona et al., 2007). In the chicken small and large intestine of older animals, low to medium mRNA levels were found for  $\beta$ -defensins, previously reported to be expressed in heterophils and/or bone marrow only (Table 2), suggesting it to originate from resident myeloid cells. Considerable AvBD13 mRNA expression was found in small intestinal tissue, liver and gall bladder (Higgs

Table 2  
Tissue-specific chicken  $\beta$ -defensin gene expression

Tissues	AvBD1	AvBD2	AvBD3	AvBD4	AvBD5	AvBD6	AvBD7	AvBD8	AvBD9	AvBD10	AvBD11	AvBD12	AvBD13
Tongue	–	–	s	–	m/s	–	–	–	–	–	–	–	–/m
Esophagus	–	–	m	–	–	–	–	–	w/s	–	–	–	–
Crop	–	–	–	–	–	–	–	–	–/s	–	–	–	–
Proventriculus	–	–	–	–	–	–	–	–	–/m	–	–	–	–/m
Gizzard	–	–	–	–	–	–	–	–	–/~	–	–	–	–
Small intestine	–/w	–/m	–	–/~	–	–/~	–/~	–	–/w	–	–	–	–/s
Large intestine	–/w	–/m	–/~	–/~	–	–/m	–/~	–	–/~	–	–	w	–
Caeca	m	m	–	–	–	–	–	–	–	–	–	–	–
Colon	–	–	–	–	–	–	–	–	–	–	–	–	w
Cloaca	w	–/w	–	–/~	–	–	–	–	–	–/w	–	–	–
Pancreas	w	–/w	–	–	–	~	–	–	~	–	–	–	m
Liver	–	–/w	–	–/w	–	–/w	–	m	m/s	m/s	–	–	m/s
Gall bladder	w	w	–	~	–	w	~	m	s	s	–	–	s
Trachea	–	–/w	–/s	–/w	~w	–/~	–/~	–	w/s	–	–	–	~m
Lung	m/s	m/s	–	–/w	~	–/m	–/~	–	–/w	–/m	–	–	m
Air sacs	–	–	m	–	–	–	–	–	–	–	–	–	m
Kidneys	–	–	–/w	–/~	–	–/w	–	–	m/s	m/s	s	w	m
Testis	s	s	–	–/s	–/~	–/s	–/s	–	–/m	m/s	–	w	–
Vas deferens	–	–	–	–	–	–	–	–	m	~	–	–	–
Ovary	–	–	w	–	–	–	–	–	–/m	m	–	~	~
Oviduct	–	–	–	–	–	–	–	–	–	m	s	s	w
Infundibulum	m	m	m	–	–	–	–	–	–	m	–	–	–
Uterus	–/w	–/w	~	–	–	–	–	–	–	w	w	s	–
Vagina	m	w	m	–	–	–	–	–	–	–	–	–	~
Egg yolk	–	–	–	–	–	–	–	–	–	m	–	–	–
Skin	–	–	–/m	–	–	–	–	–	–/m	–/w	m	–	–
Thymus	–	–	–	–	–	–	–	–	–/w	–	w	–	–
Spleen	–	–/w	–	–	–	–	–	–	–/w	–	–	–	–/s
Bursa	–/m	–/m	s	–/w	–/w	–/m	–/~	–	w/s	~w	–	m	~m
Heart	–	–	–	–	–	–	–	–	–	–	–	w	–
Skeletal muscle	–	–	–	–	–	–	–	–	–/m	–	–	–	–
Brain	w	w	~	–/~	~w	–/~	–/~	–	w/m	–/~	–	–	–
Bone marrow	s	s	w	m/s	w/s	s	s	–	w	–	–	–	–
Leukocytes	s	s	–	–	–	w	w	–	–	–	–	–	–
References	Harwig et al. (1994), Lynn et al. (2004), Ohashi et al. (2005), Zhao et al. (2001) and Sadeyen et al. (2004)	Harwig et al. (1994), Lynn et al. (2004), Ohashi et al. (2005), Zhao et al. (2001) and Sadeyen et al. (2004)	Lynn et al. (2004), Ohashi et al. (2005), and Zhao et al. (2001)	Lynn et al. (2004) and Xiao et al. (2004)	Lynn et al. (2004) and Xiao et al. (2004)	Lynn et al. (2004) and Xiao et al. (2004)	Lynn et al. (2004) and Xiao et al. (2004)	Lynn et al. (2004) and Xiao et al. (2004)	Lynn et al. (2004), van Dijk et al. (2007) and Xiao et al. (2004)	Lynn et al. (2004) and Xiao et al. (2004)	Xiao et al. (2004)	Lynn et al. (2004) and Xiao et al. (2004)	Higgs et al. (2005) and Xiao et al. (2004)

Expression levels: (s)trong, (m)oderate, (w)eak, ~ trace, – not detected.

et al., 2005). Similarly, moderate to high mRNA expression in liver (and gall bladder) was found for AvBD8, -9, and -10 (Higgs et al., 2005; Lynn et al., 2004; Xiao et al., 2004), which may reflect an important role of avian  $\beta$ -defensins in the liver during systemic infections. Moderate AvBD1 and -2 expression was detected by real-time PCR in caecal tissue of 3–7-week-old (Sadeyen et al., 2004) and 30-week-old chickens (Sadeyen et al., 2006). Other studies on avian  $\beta$ -defensin expression did not include caecal tissue. Low levels of AvBD13 mRNA were found in colon (Xiao et al., 2004) and only weak to moderate mRNA expression levels were found for AvBD1, -2 and AvBD10 in the cloaca (Lynn et al., 2004). The cloaca and colon are a point of entry for potential micro-organisms as in birds anti-peristalsis of the lower intestine, the so-called intestinal reflux, is capable of transporting faeces back into the intestine and past the ileocaecal junction (Duke, 1986). The caecal pouches are the main fermentation sites of poorly digestible substrates and are emptied only once every 8 h on average. Thus, an efficient local immune barrier can be expected to be present at this site to prevent or limit pathogen invasion in the intestinal and urogenital tracts via this infection route.

### 5.5. Urogenital tract

In birds, the cloaca is also the collecting point of the urogenital tract. Ohashi et al. (2005) examined AvBD1, -2 and -3 mRNA expression in the hen reproductive tract by semi-quantitative RT-PCR and showed that the highest levels occurred in infundibulum for all three gallinacin genes and in vagina for AvBD1 and AvBD3. Localization of expression sites in vaginal tissue using *in situ* hybridisation identified AvBD1, -2 and -3 in basal cells of the surface epithelium in the mucosal folds. The onset of egg-laying activity at approximately 18 weeks of age (Wigley et al., 2005) and absence of significant AvBD1 to -3 expression in the oviduct reported in 3-month-old hens by Zhao et al. (2001) suggest that expression levels of these gallinacins in the oviduct may be developmentally affected by estrogen levels. AvBD1, -2 and -3 mRNA levels were significantly higher in the vaginal mucosa of older birds, i.e. 180-day-old versus 720-day-old hens (Yoshimura et al., 2006). The decreased avian  $\beta$ -defensin levels found in the regressed oviducts of feed-withdrawal-induced non-laying birds further supports the idea of fluctuating avian  $\beta$ -defensin expression levels as a function of egg-laying activity regulated by gonadal steroid hormone levels. Stimulation of cultured chicken

vaginal cells with *S. enteritidis* or LPS increased levels of AvBD1 to -3 within 24 h. The importance of  $\beta$ -defensins in the protection of the mammalian male and female reproductive tracts is well established. Abundant expression of multiple  $\beta$ -defensin genes in the male (Palladino et al., 2003; Patil et al., 2005; Sang et al., 2005, 2006; Yamamoto and Matsui, 2002) and female (Aono et al., 2006; Quayle et al., 1998; Valore et al., 1998) reproductive tracts has been reported. The male reproductive system is largely devoid of an adaptive immune system and is therefore depending on an effective innate immune system to prevent infection that may affect temporary or permanent fertility (Patil et al., 2005). Mammalian sperm cells are terminally differentiated when they leave the testis, but lack motility and are incapable of fertilization. During their migration through the epididymis, spermatozoa undergo physiological and functional maturation as a result of which they acquire forward motility and the ability to recognize the zona pellucida (Lakoski et al., 1988). Several mammalian species have been shown to possess chromosomally clustered  $\beta$ -defensin genes which are differentially expressed, predominantly or restricted, throughout the epididymis (Yamaguchi et al., 2002; Yenugu et al., 2006) and transcriptionally regulated by androgens or other testicular factors (Oh et al., 2006; Yenugu et al., 2006). Epididymal  $\beta$ -defensins have demonstrated to induce sperm motility and are involved in the sperm capacitation process (Yudin et al., 2003, 2005; Zhou et al., 2004). It is thought that they may either form  $\text{Ca}^{2+}$  permeable channels or activate L-type  $\text{Ca}^{2+}$  channels in sperm cells, as a result of which  $\text{Ca}^{2+}$  could accumulate and induce motility and capacitation (Zhou et al., 2004). In chicken several  $\beta$ -defensins were found to be strongly expressed in testis (AvBD1, -2, -4, -6, -7 and -10), whereas weak expression levels were observed for AvBD12 in testis and moderate expression levels were found for AvBD9 in testis and vas deferens (Table 2). Expression levels of  $\beta$ -defensins in epididymal tissue have not yet been addressed. A recent report indicates that human  $\beta$ -defensin-2 expression contributes, in cooperation with resident flora, to protection against vaginal infection (Valore et al., 2006). The high mRNA levels of multiple avian  $\beta$ -defensins in kidney and throughout the male and female reproductive tracts (Table 2) suggest a similar role for avian  $\beta$ -defensins in the protection of the avian urogenital tract. The role of  $\beta$ -defensins in avian sperm maturation and capacitation and existence of avian epididymis-specific  $\beta$ -defensins remains to be elucidated.

The contrast in  $\beta$ -defensin expression levels between bursa of Fabricius, spleen and thymus,

bursa  $\gg$  spleen  $>$  thymus, can be explained by their localization. The bursa is located near the cloaca and therefore continuously exposed to microorganisms. It is therefore likely that the local moderate to high expression of several avian  $\beta$ -defensins in this organ, aids to its protection against pathogenic microorganisms.

In summary, the cloacal region harbors the site of B-cell generation, collects urine, and at the same time forms an important junction of the intestinal and reproductive tract. Therefore, it would be very interesting to investigate the role of  $\beta$ -defensins in the local innate immune defense of this region.

### 5.6. Other species

Apart from studies involving the king penguin (*Aptenodytes patagonicus*)  $\beta$ -defensins, little is known about the repertoire and functions of avian  $\beta$ -defensins in wild birds. Although  $\beta$ -defensin sequences related to known chicken  $\beta$ -defensins have been found for king pigeon (*Columba livia*, AvBD4) and mallard duck (*Anas platyrhynchos*, AvBD2, -9) and have been deposited in the Genbank database (Table 1), no related functional data have been published. Recently, AvBD4 related  $\beta$ -defensin sequences have been found in gastrointestinal tissues of the blue tit (*Parus caeruleus*), herring gull (*Larus argentus*) and wood pigeon (*Columba palumbus*) (Milona et al., 2007). Reverse transcriptase PCR analyses detected expression of AvBD4-related sequences in all three non-domesticated species, with highest expression levels in wood pigeon gizzard, and additional low expression in small intestine and liver. For herring gull, low levels were found in small intestine, gizzard and liver, whereas in the blue tit, low AvBD4-like expression was observed in small intestinal tissue, but not in gizzard. Comparative research in different birds from diverse habitats and under various conditions may contribute to our understanding of the functions of avian defensins.

### 5.7. Induction of avian $\beta$ -defensin expression

Whereas  $\beta$ -defensin genes may be constitutively expressed in some tissues, their expression can be upregulated in other tissues in response to microbial infection or by proinflammatory stimulants. In mammals,  $\beta$ -defensins have shown to be expressed by peripheral blood cells, dendritic cells, keratinocytes, and the epithelial cells lining the respiratory, gastrointestinal and urogenital tracts (Bals et al., 1999; Duits et al., 2002; Ohara et al., 2004; Oren et al., 2003) and to

be induced or upregulated by cytokines IL-1 $\alpha$  (O'Neil et al., 2000), IL-1 $\beta$  (Singh et al., 1998), TNF- $\alpha$  (Harder et al., 2001), IFN- $\gamma$  (Duits et al., 2002), TGF-1 and insulin-like growth factor 1 (Sørensen et al., 2003), LPS (Fang et al., 2003), bacteria (Fang et al., 2003; Harder et al., 2001; O'Neil et al., 2000; Veldhuizen et al., 2006), yeast (Pivarsci et al., 2005) and other stimulants such as PMA (Krisanaprakornkit et al., 2000), isoleucine (Fehlbaum et al., 2000) and 1,25-dihydroxyvitamin D<sub>3</sub> (Wang et al., 2004).

Induction also seems to be the case for the avian  $\beta$ -defensins, as seen for AvBD3, which was significantly upregulated in tracheal tissue of *Haemophilus paragallinarum*-challenged animals, but not in other tissues (Zhao et al., 2001). The presence of transcription factor binding sites known to be involved in mammalian  $\beta$ -defensin regulation in the chicken AvBD9 promoter region and the observation of highly variable AvBD9 levels in crop tissue of 13-day-old chicken broilers, indicate a possible tissue-specific upregulation of the AvBD9 gene (van Dijk et al., 2007). Small intestinal AvBD4, -5 or -6 mRNA levels were not upregulated in response to an oral challenge with *Salmonella* serovars (Milona et al., 2007).

Because tissue-specific  $\beta$ -defensin expression and upregulation might be breed-dependent, Sadeyen et al. (2006, 2004) investigated the relationship between this and gene expression of innate immune response factors in *S. enteritidis* carrier state. Two inbred chicken lines differing in resistance to caecal colonization by *S. enteritidis* have been described (Bumstead and Barrow, 1988). In both young and adult animals, AvBD1 and AvBD2 levels were indeed higher ( $\sim 10$ -fold) for the 6<sub>1</sub> line (resistant phenotype) as compared to the 15I line (susceptible phenotype), which would indicate a possible relation between defensin levels and *Salmonella* carrier status. However, young animals of the 6<sub>1</sub> line actually had higher bacterial loads in the caeca (Sadeyen et al., 2004), which suggests that elevated AvBD1 and -2 levels were not directly responsible for the increased resistance against *Salmonella* caecal carrier status.

In male king penguins (*A. patagonicus*), gastric  $\beta$ -defensin expression can be upregulated when fasting during the breeding season (Thouzeau et al., 2003). King penguins only feed at sea, which can entail a 400–500 km journey, and while on land they must live off their reserves. After egg-laying, females return to sea to forage and egg-incubation is taken over by the male. Usually, females come back in time to feed the chick at hatching, but their mates cope with a delayed return of their partner by fasting, thus preserving food in their

stomach for 2–3 weeks, enabling them to feed the newborn chick for about 10 days (Gauthier-Clerc et al., 2000). Analysis of the stomach contents of male penguins identified two  $\beta$ -defensins, AvBD103a and AvBD103b and other yet unidentified antimicrobial substances (Thouzeau et al., 2003). AvBD103a and -103b concentrations in the stomach contents were compared between food conserving and normally digesting birds during the egg-incubation period. AvBD103b was detected in the stomach of all birds, whereas AvBD103a was detected in only three samples of one conserving bird (Thouzeau et al., 2003). Stomach  $\beta$ -defensin concentrations were markedly higher in food conserving birds than in digesting birds, i.e. increasing 13-fold from the onset (74 nM) to the end of the fast period (943 nM). Defensin levels in digesting birds remained invariably low (24 nM). By comparison, in mammals defensin concentrations of more than 3 mM have been found in the granules of mammalian leukocytes (Ganz, 1987; Ganz et al., 1985), whereas the concentration of cryptdins (released by Paneth cells) in the crypt lumen was estimated to be  $\sim$ 2.4 mM (Ayabe et al., 2000). Approximately 4.5–23  $\mu$ M of porcine  $\beta$ -defensin-1 was found in pig dorsal tongue scrapings (Shi et al., 1999).

Comparison of the published data from different research groups (Table 2) show that avian  $\beta$ -defensin expression levels are highly variable. It should be noted though that the reported expression levels of avian  $\beta$ -defensins are almost solely based on reverse transcriptase PCR data, which provides at the most a semi-quantitative estimation of tissue mRNA levels. Besides differences in used breeds, animal age and immune status, tissue-specific gene expression levels may even vary considerably between individual animals. Therefore, determination of actual peptide levels in healthy and challenged animals *in situ* may shed some light on their local biological importance in immune homeostasis and response to infection.

## 6. Antimicrobial activity

The few avian  $\beta$ -defensins that have been studied for their antimicrobial activities display a wide range of microbicidal or microbistatic activities against Gram-negative and Gram-positive bacteria, and fungi (Table 3). It should be noted that the MIC values mentioned in this section, are highly dependent on the type of assay, incubation medium and incubation time used.

Evans et al. (1995) demonstrated bactericidal and fungicidal activity of chicken and turkey heterophil AvBD1, at peptide concentrations of 0.4–3.4  $\mu$ M and

0.4–1.8  $\mu$ M, respectively, against avian pathogens. However, these peptides were not able to kill *P. multocida* or neutralize Infectious Bronchitis Virus, an enveloped coronavirus of chickens. A (20 aa) fragment of turkey AvBD2 inhibited the growth of *S. aureus*, but not of *E. coli* (Evans et al., 1994). Synthetic chicken AvBD9 peptide showed strong microbicidal activity against the Gram-negative bacterium *C. jejuni* (3.7  $\mu$ M), Gram-positive bacteria, *C. perfringens*, *S. aureus* (1.9–3.7  $\mu$ M) and the yeasts *C. albicans* and *S. cerevisiae* (1.9  $\mu$ M), but was less potent against *E. coli* (7.5  $\mu$ M) and not bactericidal against *S. typhimurium* ( $>30$   $\mu$ M) (van Dijk et al., 2007). In contrast, synthetic chicken AvBD13 peptide was only bactericidal at high peptide concentrations against *L. monocytogenes* (114  $\mu$ M) and *S. typhimurium* wild-type (114  $\mu$ M) and a *S. typhimurium* *Pho P* mutant (57  $\mu$ M), whereas inhibition of *E. coli*, *S. aureus* and *S. pyogenes* at peptide concentrations  $\leq 57$   $\mu$ M was negligible or absent (Higgs et al., 2005).

In radial diffusion assays, ostrich heterophil  $\beta$ -defensins, AvBD1, -2, and -7, efficiently inhibited the growth of *E. coli* O157:H7 and methicillin-resistant *S. aureus* strain 1056 (MRSA) with MICs ranging from 0.2 to 0.6  $\mu$ M (Sugiarto and Yu, 2006). Ostrich AvBD8 was less potent against these bacterial strains (MIC, 2.4  $\mu$ M), whereas only Ostrich AvBD1 was fungicidal against *C. albicans*.

Analysis of the stomach contents of male king penguins revealed numerous antimicrobial activities, including the avian  $\beta$ -defensin peptides AvBD103a and AvBD103b, which are identical with the exception of an Arg residue instead of a His residue at position 14 for AvBD103b. Synthetic penguin AvBD103b peptide displayed potent bactericidal activity against Gram-positive bacteria (*K. rhizophila*, *Bacillus* spp., *Staphylococcus* spp., *N. asteroides* and *A. viridans*), with the exception of *S. saprophyticus*, at peptide concentrations less than 4  $\mu$ M (Thouzeau et al., 2003). Mainly bacteriostatic activity was observed for AvBD103b against Gram-negative bacteria, although it displayed bactericidal activity against an *E. coli* strain. In contrast to its impotence against *Candida glabrata* ( $>100$   $\mu$ M) and *Candida albicans* (50–100  $\mu$ M), the yeast *Candida tropicalis* and filamentous fungi *Neurospora crassa* and *Aspergillus fumigatus* were efficiently killed (3–6  $\mu$ M) by AvBD103b.

## 7. Mechanisms of action

Mature  $\beta$ -defensin peptides have a three-dimensional amphipathic structure, i.e. they possess spatially

Table 3  
Antimicrobial activity of avian  $\beta$ -defensins

	Microorganisms	Chicken AvBD1 (Evans et al., 1994, 1995; Harwig et al., 1994)	Chicken AvBD2 (Evans et al., 1994; Harwig et al., 1994)	Chicken AvBD9 (van Dijk et al., 2007)	Chicken AvBD13 (Higgs et al., 2005)	Turkey AvBD1 (Evans et al., 1994, 1995)	Turkey AvBD2 fragment (Evans et al., 1994)	Ostrich AvBD1 (Sugiarto and Yu, 2006)	Ostrich AvBD2 (Sugiarto and Yu, 2006)	Ostrich AvBD7 (Sugiarto and Yu, 2006)	Ostrich AvBD8 (Sugiarto and Yu, 2006)	Penguin AvBD103b (Thouzeau et al., 2003)
G (–)	<i>Escherichia coli</i>	✓	✓	✓	×	✓	×	✓	✓	✓	✓	>,✓
	<i>Salmonella enteritidis</i>	✓				✓						
	<i>Salmonella typhimurium</i>	✓		✓	×	✓						>
	<i>Pasteurella multocida</i>	✓				×						
	<i>Campylobacter jejuni</i>	✓		✓		✓						
	<i>Bordetella avium</i>	✓				✓						
	<i>Klebsiella pneumonia</i>											>
	<i>Pseudomonas aeruginosa</i>			×								>
	<i>Enterobacter cloaca</i>											×
	<i>Alcaligenes faecalis</i>											×
	<i>Vibrio metshnikovii</i>											>
	<i>Vibrio anguillarum</i>											>
G (+)	<i>Listeria monocytogenes</i>	✓	✓		×							✓
	<i>Staphylococcus aureus</i>	✓		✓	×		✓	✓	✓	✓	✓	✓
	<i>Staphylococcus haemolyticus</i>											✓
	<i>Staphylococcus saprophyticus</i>											>
	<i>Streptococcus pyogenes</i>			✓	×							
	<i>Clostridium perfringens</i>			✓								
	<i>Kocuria rhizophila</i>											✓
	<i>Bacillus subtilis</i>											✓
	<i>Bacillus cereus</i>											✓
	<i>Bacillus megaterium</i>											✓
	<i>Nocardia asteroides</i>											✓
	<i>Aerococcus viridans</i>											✓
M	<i>Mycoplasma gallisepticum</i>	✓				✓						
F	<i>Candida albicans</i>	✓	×	✓		✓		✓	×	×	×	>
	<i>Candida tropicalis</i>											>
	<i>Candida glabrata</i>											>
	<i>Saccharomyces cerevisiae</i>			✓								
	<i>Neurospora crassa</i>											>
	<i>Aspergillus fumigatus</i>											>
V	<i>Infectious Bronchitis Virus</i>	×				×						

Microbicidal activity (✓), microbistatic activity (>) or no growth inhibition (×) at peptide concentrations below 10  $\mu$ M. G (–), Gram-negative bacteria; G (+), Gram-positive bacteria; M, mycoplasma; F, fungi; V, enveloped virus.

opposite domains of clustered hydrophobic and cationic aa side chains. Three intramolecular disulfide bridges restrict conformational changes of these peptides and are well-conserved in this family. For most mature defensins, disulfide bridges and their connectivity appear not to be important for direct antimicrobial activity, but may play a prominent role in other functions, such as protection against proteolysis and chemotaxis (Klüver et al., 2005; Selsted and Ouellette, 2005; Wu et al., 2003). On the other hand, amino acid composition and positioning are highly variable and appear to determine the extent to which individual  $\beta$ -defensins specifically target certain types of microorganisms (Torres and Kuchel, 2004). Their ability to inhibit growth and/or kill microorganisms differs considerably and is likely achieved via multiple mechanisms. Fig. 2 shows a hypothetical “carpet-wormhole model” of action for defensins (Ganz, 2003). Cationic peptides are able to interact electrostatically with negatively charged membrane components, such

as lipopolysaccharides (LPS), lipoteichoic acid (LTA) and anionic phospholipids and subsequently pass the membrane via the “self-promoted uptake pathway” (Hancock, 1997). Due to their higher affinity for divalent cation binding places in the outer membrane, cationic peptides can competitively displace  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  ions, important for microbial membrane stability, and subsequently by their larger size, perturb the membrane structure (Hancock, 1997; Hancock and Chapple, 1999). Driven by the large electric potential of the membrane, the perturbing peptide migrates through the membrane and aggregates into multimeric peptide clusters with their hydrophilic sides facing inwards, resulting in stable or transient pore formation or may disrupt the membrane in a detergent-like way (Ganz, 2003; Oren and Shai, 1998). Alternatively, formation of membrane regions with increased permeability, have been proposed. Although the above-described mechanisms of permeabilization, involving carpet formation and pore formation, are supported by ultrastructural

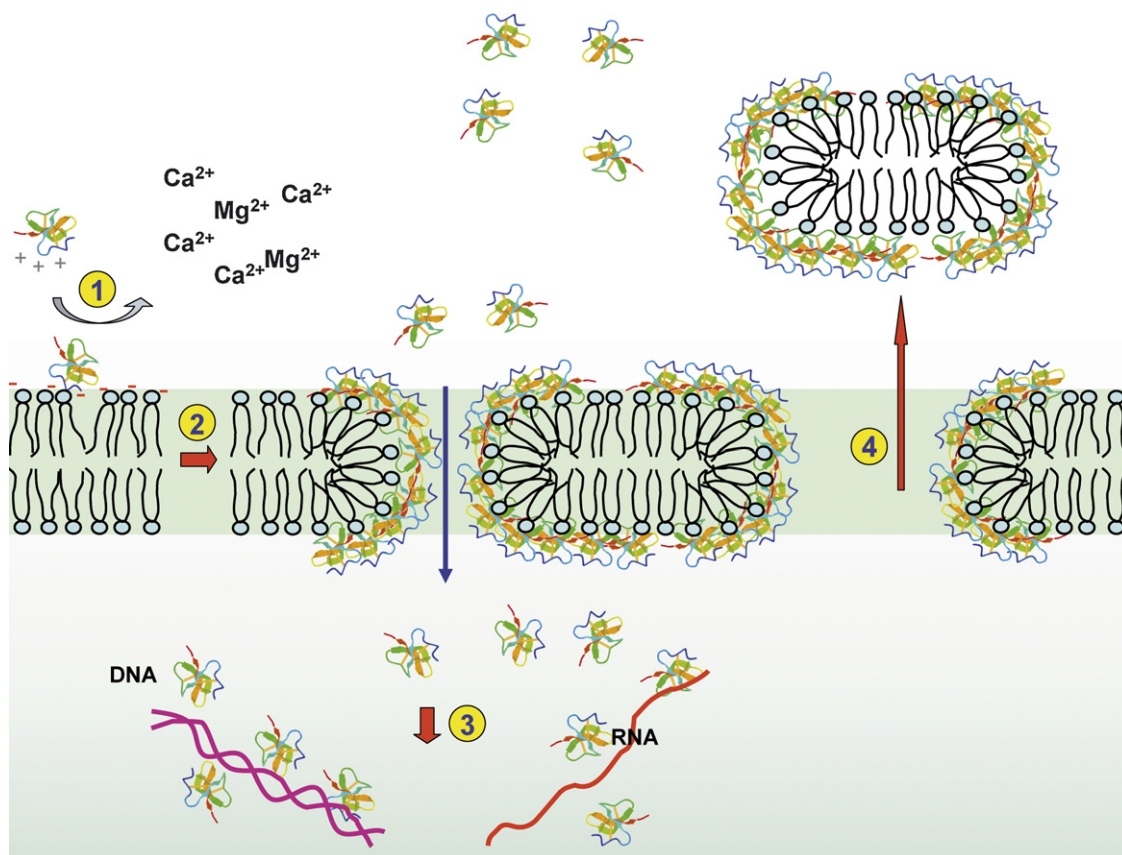


Fig. 2. Mechanisms of action of cathelicidins and defensins according to the “carpet/wormhole model”. Displacement of membrane-stabilizing ions during initial electrostatic interaction with outer membrane components (1) is followed by accumulation of peptides parallel to the membrane and formation of transient pores (2) or in detergent-like membrane disruption (4). Additionally, intracellular interactions with DNA, RNA or proteins may disable protein synthesis and function (3).

studies, this model and alternative models are primarily based on work done with artificial membranes.

At lower peptide concentrations, pore-formation results in loss of potassium and other small molecules and leads to membrane depolarization (Lehrer et al., 1989). Without a membrane proton-motive force, active transportation of substances across the membrane and rotation of bacterial flagella will stop leading to starvation, a loss of motility and, eventually, lysis. Once transported to intracellular sites, cationic peptides can interfere with DNA, RNA and protein synthesis by binding to DNA and RNA molecules (Lehrer et al., 1989; Tang et al., 1999). In addition, some defensins are able to bind to membrane glycoproteins and may be potentially important for anti-viral activity (Wang et al., 2003). Alternatively, the displacement of divalent cations in the cell wall by cationic peptides can promote autolysis by activating the autolytic cell wall enzymes (muramidases) in bacterial membranes. The main regulators of autolysins in Gram-positive bacteria are (lipo)teichoic acids (Ginsburg, 2002). In dividing Gram-positive cells, activated autolysins remodel the bacterial peptidoglycan layer by their muramidase activity (Koch, 2001). Teichoic and teichuronic acids in the peptidoglycan layer of the Gram-positive cell wall bind autolysins non-competitively and in that way inhibit muramidase activity. Cationic peptides bind to teichoic acids with a higher affinity and can thereby displace and activate autolysins leading to uncontrolled degradation of the muramidase layer and often spontaneous lysis of the cytoplasmic membrane (Bierbaum and Sahl, 1987). In fact, activation of autolysins in the cell wall of the Gram-positive bacteria has been observed in the presence of poly-L-lysine (Bierbaum and Sahl, 1985). In Gram-negative bacteria, the regulation of autolysins is not fully understood (Höltje, 1995).

The antimicrobial activity of many defensins is diminished in the presence of salts, such as sodium chloride concentration at physiological concentrations (~150 mM or 300 mOsm) or by various divalent cations or plasma proteins (García et al., 2001; Goldman et al., 1997; Singh et al., 1998). Most likely, mono- or divalent cations inhibit by simple charge competition of the initial interaction between the cationic peptide and its anionic targets (Lehrer et al., 1993). This may depend on the bacterial species studied. Chicken AvBD9-mediated growth inhibition of *E. coli* and *S. typhimurium* was not affected in the presence of 20 mM sodium chloride, whereas growth inhibition of *C. perfringens* and *S. aureus* declined to ~50%. Thus, AvBD9 proved to be relatively salt-

insensitive, as 24–49% growth inhibition was retained for most strains in the presence of 150 mM sodium chloride (van Dijk et al., 2007). Living in a salt water habitat, it was not surprising that the antibacterial activity of synthetic king penguin AvBD103b against *E. coli* and *S. aureus* was not affected by sodium chloride concentrations up to 160 mM (348 mOsm), which is close to the osmolality measured in penguin stomach contents (324 mOsm) (Landon et al., 2004). This indicates that these peptides can retain their microbicidal activity *in vivo* in stomach contents and contribute to protection against food degrading microorganisms. At 480 mM NaCl the efficacy against *S. aureus* decreased 16-fold and growth inhibition of *E. coli* decreased 2- and 4-fold in the presence of 1 and 50 mM MgCl<sub>2</sub>, respectively (Landon et al., 2004). Some  $\beta$ -defensins have the propensity to form oligomeric structures. The capacity to create stable dimers in solution has been suggested as a possible reason for the high and salt-independent antibacterial activity of hBD-3 (Schibli et al., 2002). The solution structure of AvBD103b obtained by NMR spectroscopy indicated a monomeric nature for this peptide, although the authors did not rule out the formation of a symmetrical dimer (Landon et al., 2004). Thus, despite the observations that oligomerization may enhance antimicrobial activity (Campopiano et al., 2004; Schibli et al., 2002), in the case of AvBD103b and hBD-3, high cationicity (10+ and 11+, respectively) and overall hydrophobicity (Klüver et al., 2005) appears to be more important. Despite the demonstrated inactivation of some AMPs at physiological salt concentrations *in vitro*, AMP gene products correlate well with increased antimicrobial resistance in animal model experiments (Salzman et al., 2003). A possible explanation for this phenomenon was postulated by Dorschner et al. (2006), who suggested that bicarbonate, which is ubiquitously present in blood, sweat, and the mucosal surfaces of the respiratory, urogenital, and gastrointestinal tracts, could enhance microbial susceptibility to AMP-mediated killing.

## 8. Conclusions

The biological functions of avian defensins are still largely unknown, but are expected to reflect those described for their mammalian counterparts. Many questions remain to be answered. The relationship between copy number, gene polymorphisms and defensin gene repertoire with disease resistance need to be sorted out. The occurrence of elongated defensin-like genes, due to an additional large post-piece, in some

human and avian defensins suggest biological functions other than direct antimicrobial activity for these molecules. Evidence is mounting that heterophils play an important role in avian innate immune defense against bacterial infections, but their defensin repertoire and the role of their extracellular and intracellular release, subsequent activation and regulation needs to be further investigated. Similarly, the tissue distribution and scarce data on upregulation of avian AMP expression is almost completely based on regulation at the transcriptional level. Therefore, additional research on translational regulation and post-translational processing and modifications should be performed and coupled to functional studies with respect to tissue-specific expression. Particularly the cloaca and surrounding tissues, where reproductive tract, B cell synthesis and intestinal contents converge, is an interesting subject for further studies. Increased knowledge about poultry defensin biological functions, their localization and regulation may aid in the selection of breeds that are less susceptible to colonization of pathogenic bacteria. Alternatively, local endogenous defensin expression levels may be stimulated via dietary modulation.

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