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Molecular aspects of avian oogenesis and fertilisation

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ABSTRACT In this paper, we summarise studies which have been carried out on the metabolism of nucleic acids (maternal RNA, DNA, nucleolytic enzymes) in avian oocytes and embryos (Japanese quail*, Coturnix coturnix japonica)* within the last 10 years in the Institute of Genetics and Animal Breeding of the Polish Academy of Sciences. The accumulation of maternal RNA in the quail oocyte during oogenesis is shown and discussed. Several individual transcripts were identified in RNA from the germinal disc and some also in extraembryonic RNA under the perivitelline membrane. The presence of the transcript encoding chick zona pellucida C protein *(chZPC)* points to the possibility of ZPC synthesis by the oocyte itself. The transcript encoding AA-NAT (arylalkylamine N-acetyltransferase, the penultimate enzyme in melatonin synthesis) was present in 2 forms (with and without an intron) and the ratio of the two forms changes during oogenesis. Melatonin and the two enzymes engaged in its synthesis (AA-NAT and HIOMT) have been found in the egg yolk; their transcripts and the transcripts of the melatonin receptors mel-1a,b and c are present in RNA from the germinal discs. This suggests a possible role for melatonin in early avian development. DNases I and II activity has been detected in the germinal disc and the cytoplasmic layer under the perivitelline membrane. We propose that they participate in degradation of supernumerary sperm entering avian oocytes during polyspermic fertilisation. A hypothesis to explain the selection of a single sperm participating in the formation of the zygotic nucleus is discussed; we propose that sperm entry into the centre of the germinal disc is the essential event underlying the selection mechanism.

KEY WORDS: *maternal RNA, avian oogenesis, avian oocyte, DNase, polyspermic fertilisation*

Introduction

In last decades, the molecular studies of oogenesis and fertilisation have been focused on the eggs and embryos of *Xenopus,* mouse, *Drosophila* and the nematode *Caenorhabditis,* and occasionally fish or sea urchin. It is surprising that so little attention has been paid to the most popular and easily accessible avian egg. Perhaps researchers were hindered by the anticipated difficulties in the manipulation of such a large oocyte/egg loaded with enormous amounts of yolk, or the lack of convenient *in vitro* methods for fertilisation and oocyte/embryo culture, or maybe the relatively high financial and biological costs of the avian oocytes. Studies of animals with external fertilisation were clearly facilitated by the accessibility of experimental material. Later, the rapid development of *in vitro* maturation, fertilisation and embryo culture methods for mammals greatly contributed to the explanation of the developmental mechanisms of this group of animals. However, in recent years, the major technical problems hindering studies of developmental processes in birds, have been solved following the refinement of *in vitro* culture methods for avian embryos and oocytes (Perry, 1988; Zajchowski and Etches, 2000; Olszanska *et al.*1996; 2002;).

This review is focused on the studies of the metabolism of avian maternal RNA, gene expression in early avian development and mechanisms of polyspermic fertilisation in birds. The oocytes, eggs and embryos of Japanese quail (*Coturnix coturnix japonica*) are the main subject of presented here studies from our laboratory.

Abbreviations used in this paper: AA-NAT, arylalkylamine N-acetyltransferase; chZPC, chick zona pellucida C protein; GVBD, germinal vesicle breakdown; HIOMT, hydroxyindole-O-methyltranferase; ICSI, intracytoplasmic sperm injection; mel, melatonin; MPF, maturation promoting factor; PVM, perivitelline membrane; PRBP, progesteron receptor binding protein; RNP, ribonucleoprotein; RT-PCR, reverse transcription-polymerase chain reaction; ZP3, murine zona pellucida 3 protein.

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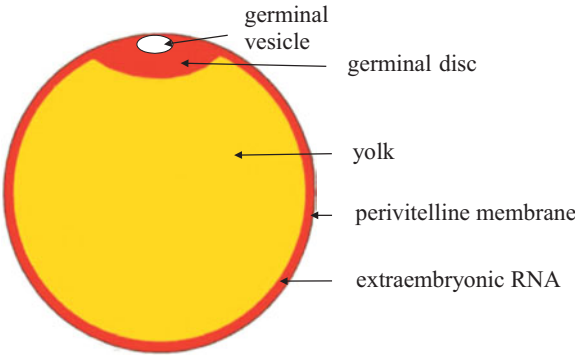

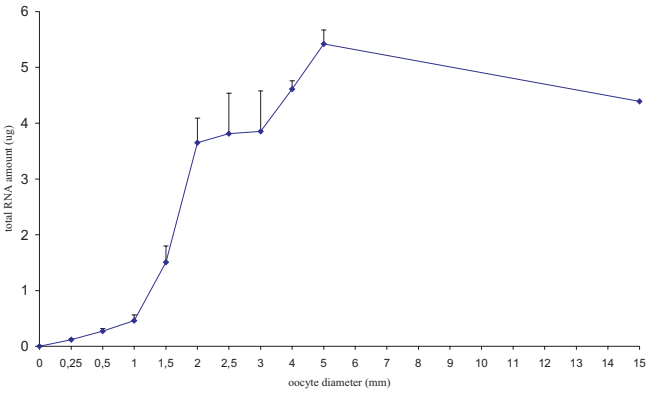

Fig. 1 (Left). Localisation of maternal RNA in the avian oocyte. *The germinal disc, the region where the embryo forms, contains RNA predestined for embryo development. The cytoplasmic layer under the perivitelline membrane contains "extraembryonic RNA", which is thought to be required for oocyte development.*

Fig. 2 (Right). Accumulation of maternal RNA in the avian oocyte *showing the amount of total RNA per one Japanese quail oocyte, measured at different stages of oogenesis.*

Maternal RNA of avian oocytes

Maternal RNA is the RNA accumulated in the oocyte during oogenesis that is used for protein synthesis during early embryonic development before the onset of the zygotic transcription from the zygotic genome.

The amount of maternal RNA in the avian oocyte is huge (~5 μg/oocyte, Malewska and Olszanska, 1999) when compared with mammals (see Table 1), although it is similar to the amount reported for *Xenopus* (~4.8-6 μg/oocyte; after Davidson, 1986). A fraction of the RNA in the avian oocyte is located in the germinal disc (1-2 μg), and this probably represents the RNA predestined for future use by the embryo. This RNA contains ~5.5% polyadenylated sequences, and is relatively stable; it is not degraded during 24h incubation at 41ºC *in vitro.* The remaining, larger fraction of the maternal RNA is located in the cytoplasmic layer underlying the perivitelline membrane (PVM) around the yolk sphere. This RNA is enriched in polyadenylated RNA (~8-9 % poly(A^+)RNA), and is degraded during the passage of the egg throughout the oviduct, before the egg is laid and also during 24h incubation *in vitro* (Table 2, Malewska and Olszanska, 1999). Due to its location outside the embryo territory, we have designated this RNA as "extraembryonic RNA" (Fig. 1). We assume that this fraction of the maternal RNA is not used by the embryo itself but might be required for the oocyte growth and development during oogenesis

Another remarkable feature of avian maternal RNA is that not all of this RNA is synthesised by the oocyte itself. In fact, the majority of this RNA is delivered to the oocyte from the outside by granulosa cells, long after the disappearance of the lampbrush chromosomes and the end of transcription in the germinal vesicle of the oocyte (Fig. 2). The curve of accumulation of total maternal RNA rises steeply in oocytes from 0.25 to 2.0 mm in diameter, plateaus in oocytes of 2.0-3.0 mm, and increases further during oocyte growth until oocyte reaches the diameter of ~5.0 mm. The first phase of intensive RNA accumulation coincides with the appearance of lampbrusch chromosomes, which for example in quail are visible in 0.25-1.5 mm oocytes (Callebaut, 1968; 1973, 1974). The subsequent plateau of RNA accumulation corre-

TABLE 1

CONTENT OF MATERNAL RNA IN THE OOCYTES/EGGS OF DIFFERENT ANIMALS

Species	Total RNA (ng)	polyA RNA (pg)	source
Strongylocentrotus purpuratus (sea urchin, eggs)	2.8	30	after Davidson, 1986
Drosophila	190	-	„ „
Xenopus (oocyte)	4 800	90	„ „
Japanese quail (oocyte)	4 500-5 500	338 - 420	Malewska & Olszanska, 1999
blastoderm, fertile laid egg	1 370	69	" "
germinal disc	1 115	63	" "
Hen germinal disc	2 100	-	Olszanska & Borgul, 1993
Guinea hen " "	1 900	-	" "
Turkey " "	1 800	-	" "
Mouse (egg)	0.3	25	after Davidson, 1986
(egg)	0.47	-	Olszanska & Borgul, 1993
Rabbit (egg)	15.0	-	" :
"	20.0	-	Manes, 1969
"	16.0	-	Schultz, 1975
Cow (oocyte)	0.98	-	Olszanska & Borgul, 1993
Pig (oocyte)	0.65	-	" "
Sheep (oocyte)	0.75	-	" "

TABLE 2

CONTENT OF MATERNAL RNA IN THE OOCYTES AND FRESHLY LAID EGGS OF THE JAPANESE QUAIL

(FROM MALEWSKA & OLSZANSKA, 1999)

Biological material	Amount of total RNA (μg) (±SD)
oocyte	
germinal disc	1.15 ± 0.06
perivitelline membrane	3.24 ± 0.03
	total 4.39
laid egg	
blastoderm	1.37 ± 0.27
vitelline membrane	0.61 ± 0.18
	total 1.98

sponds to the period of condensation of lampbrush chromosomes and inhibition of RNA synthesis in the oocyte nucleus. Thus, the second phase of RNA accumulation following the plateau (the oocytes from 3-5 mm) has to originate from the external sources. Several authors have already proposed such possibility after observing vesicular RNA-containing structures (transosomes) crossing from granulosa cells into the oocytes (Schjeide *et al.,*1970; Paulson and Rosenberg, 1972; Gorbik, 1976). It is impossible to establish how much RNA is accumulated in vitellogenic oocytes larger than 5mm in diameter because these oocytes cannot be stripped, without damage, from the follicular and granulosa cells. For this reason the level of RNA was determined for the ovulated ("naked") oocytes. It seems that at this stage the RNA accumulation stops and some degradation may even occur of existing RNA between late vitellogenesis and the ovulation stage (Fig. 2). The shape of the last part of the accumulation curve shown in Fig. 2 may be explained if one assumes that continuous delivery of RNA to the oocyte occurs in parallel with RNA degradation by nucleolytic enzymes (RNases) within the oocyte (Malewska and Olszanska, 1999). Indeed, the presence of low RNase A activity has been detected in the yolk, germinal disc and the cytoplasmic layer under a perivitelline membrane of oocytes (Stepinska *et al.*, 1996a). RNA delivery to the oocyte from the external sources ceases at ovulation or a few hours before due to the breakage of the cytoplasmic connections between the oocyte membrane and granulosa cells (Yoshimura *et al.,* 1993). The RNase may then cause degradation of extraembryonic RNA during the 24-h passage of the egg through the oviduct until oviposition, resulting in a decrease in the oocyte RNA content by ~80% (see Table 2, from Malewska and Olszanska, 1999). The degradation of this pool of RNA occurs also during 24-h *in vitro* incubation. During the same period the level of RNA in the blastoderm of a laid egg remains essentially the same as in the germinal disc, representing a stable pool of maternal RNA destined to be used by future embryo. This pool of RNA forms ribonucleoprotein complexes (RNPs) with specific proteins and is protected from degradation during *in vitro* or *in vivo* incubation for 24 h (Malewska and Olszanska, 1999). The existence of two pools of maternal RNAs in other species (mouse, *Xenopus*) has been known for many years (review by Davidson, 1986). In birds, probably as a result of the large oocyte dimensions, these RNA pools are localised in different compartments: in germinal disc and around yolk under perivitelline layer.

Presence of specific gene transcripts in the germinal disc and extraembryonic RNA

The transcript profile of oocyte and freshly laid egg RNA has been tested by Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and it is summarised in Table 3 and 4 (with some examples shown in Fig. 3). The 15 individual transcripts were always detected in the blastoderms of Japanese quail (embryonic stage 1; Hamburger and Hamilton, 1992), and some of them (mel-1b, mel-1c, c-myc, π-globin) were absent in extraembryonic egg RNA. In oocytes, most of the mRNAs (β-actin, clusterin, mel-1c receptor, progesterone receptor, PRBP, ZPC, DNase I, AA-NAT, HIOMT) were located in the germinal discs and in extraembryonic RNA. Transcripts such as mel-1a, mel-1b, c-myc and π-globin were only found sporadically (see Table 4). The presence of the globin (including π-globin) mRNAs was observed in oocytes of the chick, in spite of the fact that embryonic globin does not appear prior to stage 8 H.H., i.e. before ~30 h of incubation development in the chick (Imaizumi-Scherrer T. *et al.,* 1989). The sporadic presence of c-myc mRNA in quail germinal discs and its complete absence in extraembryonic oocyte RNA was rather surprising

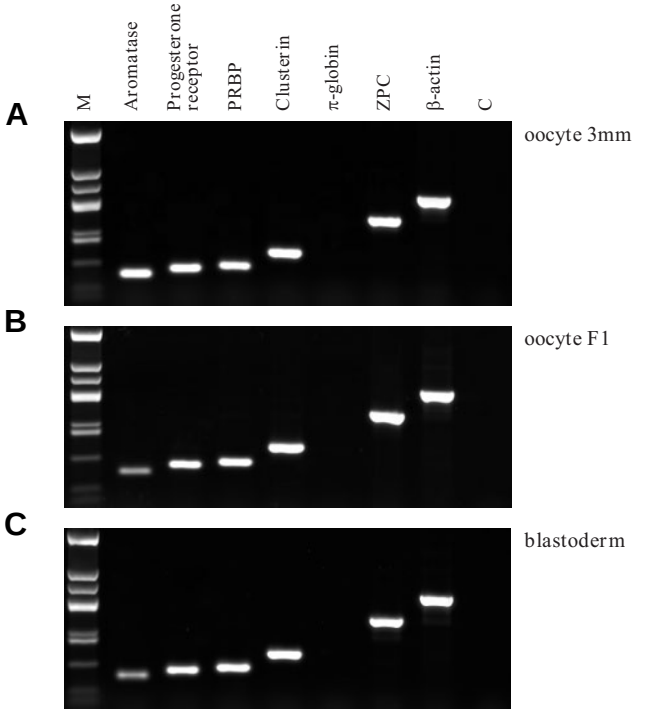

Fig. 3. Expression of selected transcripts *in previtellogenic oocytes* **(A)**, *mature oocytes* **(B)** *and in blastoderms from freshly laid eggs* **(C)**.

TABLE 3

LIST OF TESTED GENE TRANSCRIPTS

Sequence	Species	Accession no*	Length of the product PCR	RT-PCR
β-actin	hen	X 00 182	1 138	479
clusterin	Japanese quail	X 80 760	277	200
3 melatonin receptors:	hen			
mel-1a		U 31 820	517	517
mel-1b		U 30 609	259	259
mel-1c		U 31 821	372	372
estrogen receptor α	Japanese quail	AF442965	n.d.	189
estrogen receptor β	Japanese quail	AF045149	n.d.	165
progesteron receptor	hen	Y 00 092	967	206
PRBP* *	hen	U 95 088	1288	159
c-myc	hen	J 00 889	111	111
π- globin	hen	V00408/J00856	1151	278
ZPC* *	Japanese quail	ABO 12 606	750-1000	338
DNase I	hen	ABO 13 754.1	478	478
AA-NAT	Japanese quail	AF 007 068	323	238
HIOMT	Japanese quail	AF 370 034	n.d.	226

*) www.ncbi.nlm.nih.gov/entrez

**) PRBP – progesteron receptor binding protein

ZPC – chick zona pellucida – protein corresponding to the mouse zona pellucida 3 (ZP3), but also found in hen and Japanese quail (Waclawek et al., 1998).

n.d. – not determined

since the c-myc transcript is very abundant (10^5-fold higher than in somatic cells) in RNA of *Xenopus* oocytes (Taylor *et al.,* 1986).

It seems that the large avian oocyte represents a basket, where many RNA transcripts are collected during oogenesis. Some of them are synthesised by the oocyte on the lampbrush chromosomes, while other are delivered from outside by granulosa cells (the extraembryonic RNA around the yolk). At maturation/fertilisation a portion of the RNA that is not required for the immediate use undergoes degradation, after being deadenylated by a poly(A)-degrading enzyme present in the cytoplasm and nuclei of germinal discs of vitellogenic oocytes (Stepinska and Olszanska, 1996b).

Is chZPC synthesised only in granulosa cells?

Chick Zona Pellucida C protein (chZPC) is an homologue of murine ZP3, an important component of the perivitelline membrane (PVM), that serves as a structural support for the membrane, providing a receptor sites for sperm binding and triggering the acrosome reaction during fertilisation (Waclawek *et al.*, 1998). In a study using immunocytochemistry, Western and Northern blotting Waclawek *et al.* (1998) found that chZPC is produced solely in avian granulosa cells then secreted and deposited around the PVM, in contrast to mouse or fish where this protein is synthesised in the oocyte itself or in the liver, respectively. However, these authors tested the presence of ZPC transcripts in granulosa cells originating from the ovarian follicles of different stages of oogenesis but not in the oocytes. Thus, the possibility of the synthesis of ZPC in avian oocyte itself, in addition to its synthesis by the granulosa cells, cannot be excluded.

Our results using RT-PCR demonstrated the presence of chZPC transcripts in oocytes and laid eggs of quail, both in the germinal disc/blastoderm and extraembryonic RNA (Fig. 3; Table 4). However, the ZPC transcripts identified in the extraembryonic RNA under the perivitelline layer probably originate from the surrounding granulosa cells that secret the RNA into the oocyte. Although the presence of the ZPC transcript does not necessarily proves the expression of ZPC protein, it is very probable that the synthesis of ZPC protein occurs in oocyte as well as in the granulosa cells. The rapid increase in the oocyte surface during vitellogenesis justifies the need for the localization of extraembryonic RNA on the oocyte surface, around the yolk under the perivitelline membrane. In quail, within approximately 10 days of vitellogenesis the oocyte diameter increases from ~1 mm to ~20 mm, with ~400-fold increase in its surface. This means that the material building the oocyte membrane must be rapidly deposited around the yolk, and additional synthesis of membrane proteins might be expected at the oocyte surface. If this scenario is true then the extraembryonic RNA should be enriched in transcripts encoding membrane proteins. Currently, we can only say that total RNA from this compartment is enriched in polyadenylated RNA when compared to the embryonic RNA from germinal disc (~9 % versus ~6%, respectively) (Malewska and Olszanska, 1999).

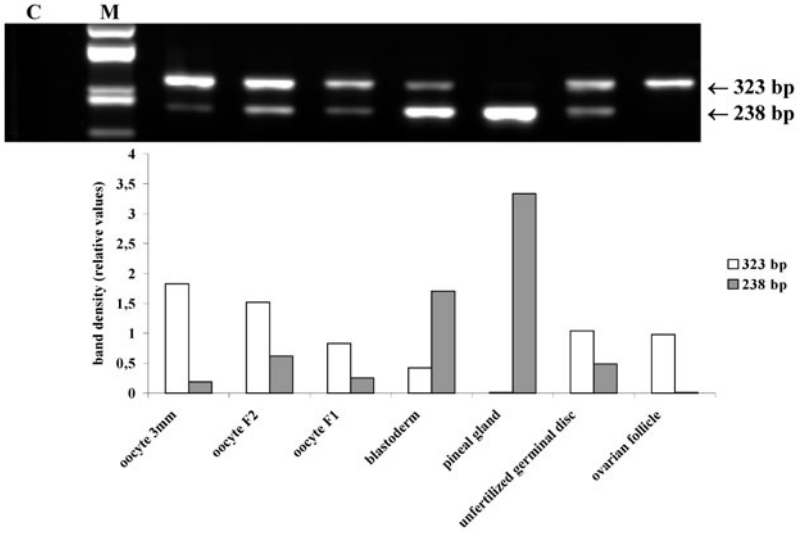

Fig. 4. Expression of two arylalkylamein N-acetyltransferase (AA-NAT) transcripts in avian oocytes and embryos, in the pineal gland and ovarian follicle. *The relative densities of the bands (RT-PCR products) are shown in the lower graph: white bars, 323-bp band (including the 85-bp intron); grey bars, 238- bp band, without intron. M, DNA size marker; C, control (no template).*

Developmental regulation of some transcripts during quail oogenesis and embryonic development

During oogenesis and embryonic development of quail, some transcripts were found to exist in different forms. We have studied the case of arylalkylamine-N-acetyltransferase (AA-NAT), a penultimate enzyme in melatonin synthesis, which is responsible for acetylation of serotonin to acetylserotonin in the pineal gland. The transcripts encoding this enzyme were examined in quail oocytes by RT-PCR method (Oblap and Olszanska, 2003). In the pineal gland, the AA-NAT transcript is present in the canonical spliced form, and RT-PCR, performed with a set of specific primers, amplified a fragment of 238 bp. However, in the oocyte, the same primer pair produced an additional product of 323 bp containing an 85-bp intron. In younger oocytes (~3 mm in diameter) this longer 323-bp product was predominant. In larger oocytes, and in the blastoderm, the abundance of this unspliced transcript decreased with a parallel increase of the shorter spliced form (Fig. 4). We also observed a similar situation during ontogenic development of the pineal gland (Oblap and Olszanska, 2004). Both transcripts appeared in the primordial pineal gland (3rd day of incubation), they persisted in the embryonic pineal gland until day 8th-9th of incubation and then the longer unspliced transcript disappeared and only the shorter, mature form of the transcript remained until hatching. Initially we assumed that the shorter intronless transcript represented the mature, translationally active form, while the larger unspliced variant was an immature form present in maternal RNA which is known to contain some unusual (repetitive, palindromic, intron) sequences interspersed with coding but translationally inactive sequences (Davidson, 1986). However, this is probably not true because both forms of the transcript were also found in quail embryos of stage 3-8 H.H. and in the brain, eye, heart and legs of the 3-day old quail embryo (Stage 21 H.H), where the presence of maternal RNA is unlikely. The single, intron-containing form of the transcript was found in the ovarian follicle. Thus, in the pineal gland and ovarian follicle, different forms of the tran-

TABLE 4

PRESENCE OF INDIVIDUAL mRNAs IN THE OOCYTES AND LAID EGGS OF THE JAPANESE QUAIL

Material	Germinal disc	extraembryonic RNA (oocyte)	Blastoderm	Extraembryonic RNA (laid egg)
Sequence				
β-actin	+	+	+	+
clusterin	+	+	+	+
melatonin receptors:				
mel-1a	-/+	-	+	+
mel-1b	-/+	-/+	+	-
mel-1c	+	+/-	+	-
oestrogen receptor α	+	n.d.	+	n.d
oestrogen receptor β	+	n.d.	+	n.d.
progesteron receptor	+	+	+	+
PRBP*	+	+	+	+
c-myc	+/-	+/-	+	-
π-globin	+/-	-	+/-	-
ZPC*	+	+	+	+
DNase I	+	n.d.	+	n.d.
AA-NAT **	+	+	+	n.d
HIOMT ***	+	+	+	n.d

(+) present; (-) absent ; (+/-) present sporadically; (n.d.) not determined .
*) as in Table 3.
**) Arylalkylamine-N-acetyltransferase – penultimate enzyme in melatonin synthesis from serotonin (two transcripts in germinal disc and in blastoderm; one (with intron) – in extraembryonic RNA).
***) Hydroxyindole-O-methyltransferase – final enzyme in melatonin synthesis

script are present: intronless and intron-containing, respectively (Fig. 4). At present the functionality of this longer intron-containing AA-NAT transcript, and why this is the only form found in ovarian follicle, are unknown. In addition, since the primers used for RT-PCR span only the 3rd intron (closest to the 3' end) we do not know whether this longer form of the AA-NAT transcript contains the two other introns present in AA-NAT gene (Oblap and Olszanska, 2003). The longer intron-containing form is most probably the unspliced mRNA and the appearance of the shorter mature transcript is likely to be due to the activation of the splicing mechanisms in the vitellogenic oocytes since the transcription on the lampbrush chromosomes is already shut off in the oocyte.

As mentioned earlier, the mel-1c melatonin receptor transcript was always detected in the maternal RNA of quail ovulated oocytes. This can be explained by preferential transcription of mel-1c gene during oogenesis or selective degradation of mel-1a and mel-b transcripts during process of maturation. Presently, we know that the second case is true and all 3 melatonin receptors are expressed in small vitellogenic oocytes, but only mel-1c is left in the ovulated ones (Kawashima et al., 2008). However, the transcripts of mel-1a and mel-1b receptors appeared later in the RNA of the blastoderms in freshly laid eggs (Fig. 5). This indicates that transcription of the other two receptor genes: mel-1a and mel-1b starts after fertilisation, during the passage of the embryo through the oviduct (Oblap and Olszanska, 2001).

Presence and possible role of serotonin and melatonin in early avian development

Melatonin is a multifunctional molecule, which, in vertebrates, is synthesised and secreted mainly, but not exclusively, in the pineal gland during the night. For a long time it has been accepted as a hormone mediating circadian and seasonal processes in animals, including reproduction. In addition, it is now recognised that the melatonin is a potent free-radical scavenger (Reiter *et al.*, 2000) and may also be regarded as an antioxidant vitamin, a tissue factor and a signalling molecule (Kvetnoy, 1999; Reiter *et al.*, 2002, Tan *et al.*, 2003). The presence of melatonin receptor transcripts in maternal RNA in oocytes, and in early quail embryo, prompted us to examine the presence and role of melatonin in early avian embryos.

So far, the role of melatonin in early embryonic development has not been studied. Mammalian embryos are permanently under the influence of melatonin circulating in mother. In contrast, the avian embryo, which is separated from the maternal organism from the early stages of development, has to be self-sufficient in respect to all nutritive and biologically active substances. Thus, if melatonin is to play any role in avian embryonic development it should either be accumulated in the oocyte or synthesised very early by the embryonic cells. The earliest melatonin synthesis was detected in pineal cells isolated from 10- and 13-days old chick embryos (Akasaka *et al.*, 1995; Moller and Moller, 1990), and this corresponds to the stage when the embryonic pineal gland acquires its vesicular structure (Jove *et al.*, 1999). The presence of serotonin – the substrate of melatonin synthesis – in chicken yolk has been reported previously (Emmanuelsson *et al.*, 1988; Moudgal *et al.*, 1992). We found that melatonin is present in quail yolk and albumen (67.5 pg/g and 22 pg/g, respectively), reaching the level of ~415 pg per egg (Olszanska *et al.*, 2007). We also detected the presence of the enzymes responsible for melatonin synthesis (AA-NAT and HIOMT) in quail yolk, and their transcripts were identified in quail germinal discs and blastoderms (Oblap and Olszanska, 2003; Olszanska *et al.*, 2007). These findings

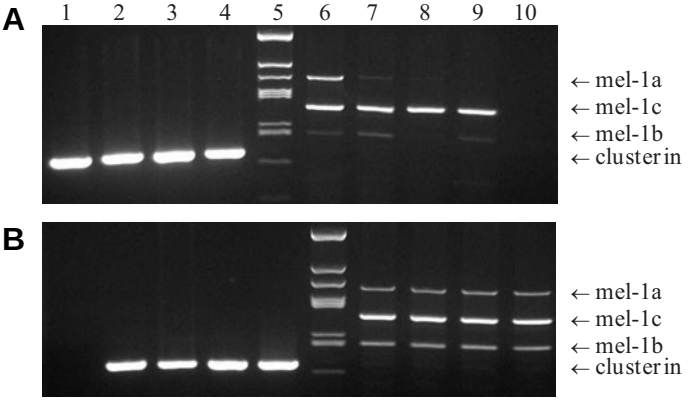

Fig. 5. Expression of transcripts encoding the melatonin receptors mel-a,b and c in quail oocytes and blastoderms. *Gel electrophoresis of the RT-PCR products using the RNA from* **(A)** *oocytes and* **(B)** *blastoderms. Lanes 1-4 (in A) and 2-5 (in B), clusterin product from RNA of 4 individual oocytes/ blastoderms; lane 5 (A) and 6 (B), DNA size marker; lanes 6-9 (A) and 7-10 (B), melatonin receptor products (mel-1 a,b,c) from the RNA of the same 4 oocytes/blastoderms used in 1-4; lane 10 (A) and 1 (B), control (no template). Clusterin was used as a control transcript that verified the absence of DNA contamination in the RNA preparation. The size of the clusterin fragment showed that it lacked an intron, confirming that it was amplified from cDNA and not genomic DNA. (From Oblap and Olszanska, Zygote, 2001).*

demonstrate that melatonin is accumulated in the egg and that all the machinery necessary for its synthesis in the future embryo is already in place in the oocyte.

What might be the function of melatonin in such early embryos, well before the onset of organogenesis and pineal gland differentiation? The most obvious is its protective role against free radicals formed during the intensive metabolic activity occurring in rapidly proliferating embryonic cells; within 24 h of uterine development, the cell number increases to ~60 000 and ~40 000 in chick and Japanese quail blastoderms, respectively (Kochav *et al.*, 1980; Stepinska and Olszanska, 1983).

Another interesting possibility is the participation of melatonin in a hypothetical "diffuse neuroendocrine system" postulated by Kvetnoy (1999), which controls communication between cells before differentiation of neural and hormonal systems. The role of serotonin, serotonin-like substances, noradrenaline and dopamine, in the regulation of some developmental processes such as cell proliferation, migration and differentiation, has been observed in sea urchin, mouse and other vertebrates (Buznikov *et al.*, 2001; Lauder 1993; Lauder *et al.*, 2000; Renaud *et al.*, 1983; Sari and Zhou, 2003; Weiss *et al.*, 1998). Serotonin was also reported to participate in the formation of the left/right axis in chick and frog embryos (Fukumoto *et al.*, 2005). However, the available data are not always unequivocal. The study of Sari and Zhou (2003) showed a concentration-dependent stimulatory effect of serotonin on the proliferation of mouse foetal heart cells, while Ilkova *et al.* (2004) found the opposite i.e. an inhibitory effect of serotonin on the cell proliferation in preimplantation mouse embryos. The antiproliferative effect of melatonin on malignant tumour cells has been frequently reported (e.g. Cos *et al.*, 2001; Jones *et al.*, 2000; Sauer *et al.*, 2001). However, the stimulation of cell proliferation and embryo development in zebrafish (Danilova *et al.*, 2004) and increased *in vitro* fertilisation and enhanced embryo development in mouse (Ishizuka *et al.,* 2000) have been also reported. Our results showing the presence of melatonin in the yolk and its possible synthesis during early avian development suggest that melatonin - a derivative of serotonin - may also participate in a signalling system based on the subtle antagonistic effects of these two substances

DNase I and II activities in the germinal disc: their possible role in polyspermic fertilisation

In avian egg, in contrast to mammals, there is no block of polyspermy triggered by the entry of first sperm, and usually many sperm enter each egg during physiological fertilisation. As a consequence, in birds, many male pronuclei are formed in the egg cytoplasm but only one that is located in the centre of the germinal disc is selected to form the zygote nucleus. The remaining surplus nuclei disperse towards the periphery of the germinal disc, and may even divide, but then they degenerate and disappear from the cytoplasm (Bekhtina, 1966; Stepinska and Bakst, 2006). Previously it was postulated that polyspermic fertilisation in birds is indispensable for the fertilisation process and normal embryo development. However, this was recently disproved by Hrabia *et al.* (2003) who achieved normal quail embryo development after *in vitro* injection of a single sperm into an ovulated oocyte (ICSI–intracytoplasmic sperm injection). We have found that in quail high level of DNase I and II activities are present in the germinal

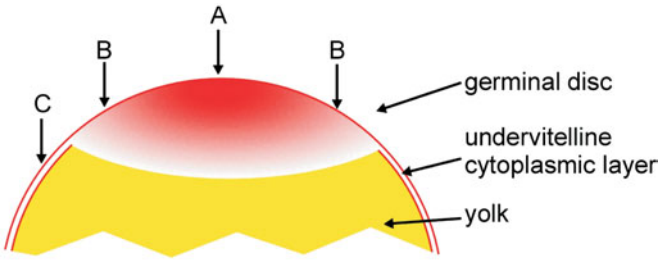

Fig. 6. Putative mechanism of selection of the single sperm destined to form the zygote nucleus during polyspermic fertilisation in birds. *(A,B,C) Points of possible sperm entry into an avian egg.* **(A)** *Region of the germinal vesicle; the single sperm entering participates in the formation of the zygote nucleus.* **(B)** *Cytoplasmic region of the germinal disc; the multiple sperm form pronuclei and may even divide but are finally degraded by DNases.* **(C)** *Non-disc region, the sperm are degraded by DNases.*

disc and in the cytoplasmic layer under the perivitelline membrane, but not in the yolk, and the sperm may be degraded by these enzymes (Stepinska and Olszanska, 2001, 2003). Such activities were not detected in mouse oocytes, which is not surprising because, in monospermic fertilisation, they would be useless or even harmful to a single spermatozoon entering the oocyte. We suggest that these DNases may participate in removal of supernumerary sperm entering the avian oocyte as a part of a specific polyspermy-preventing mechanism that is different from that operating in mammals. However, the question remains: what is the mechanism preventing the single spermatozoon, destined to form the zygote nucleus, from being degraded by the DNases? We propose that the fate of a sperm may depend on its point of entry into the germinal disc (Fig. 6, after Stepinska and Olszanska, 2003). At maturation, the germinal vesicle acquires a discoid form adhering strictly to the perivitelline layer. After extrusion of the first polar body, the second metaphase plate is located directly under the surface of the germinal disc. At this point, germinal vesicle breakdown (GVBD) has already occurred and the nuclear envelope has disappeared leaving the naked karyoplasm (Bekhtina, 1966; Yoshimura *et al.,* 1993). Therefore, a spermatozoon penetrating the perivitelline layer at the position of the former nucleus has no contact with the cytoplasm and directly enters karyoplasm, thus avoiding digestion by nucleases. In support of our hypothesis are observations of the presence of the holes in the perivitelline membrane formed by sperm entering avian oocytes in the region of the germinal disc (Bramwell and Howarth, 1992; Birkhead *et al.,* 1994). At the position of the germinal vesicle in the centre of the disc a round circle containing few sperm holes was visible, surrounded by a doughnut shaped circular belt of very densely packed holes (Bramwell and Howarth, 1992; Birkhead *et al.*, 1994). This indicates that the central area just above the germinal vesicle was penetrated by far fewer spermatozoa than the surrounding region of the germinal disc, which suggests a specific biological mechanism preventing the surplus sperm from entering the region of the germinal vesicle. Possibly, this reflects much lower density of sperm receptor proteins at this location. Besides, Iwao *et al.* (1990, 1993) showed that in newt eggs there is a gradient of maturation promoting factor activity (MPF). They suggest that the highest level of MPF is found at the centre of the

animal hemisphere where the zygote nucleus forms, while lower MPF activities occur in the vegetal hemisphere, where the supernumerary sperm degenerate. Mori *at al*. (1991) found that MPF is present in the germinal disc and it is absent outside of the disc. All these facts demonstrate that the central point in a germinal disc is a privileged location for formation of the zygotic nucleus.

Concluding remarks

There are still many unknowns and problems to solve in the study of avian oogenesis and fertilisation. For example, one completely pristine area is the ageing of oocytes and eggs. What molecular processes occur in the unfertilised oocyte beside changes in PVM? Would the ICSI procedure, used to overcome the outer PVM barrier (completed in the infundibulum), also work for oocytes taken from the lower part of the oviduct? What processes occur in stored fertile eggs as they lose their developmental potential with time? The function of melatonin and serotonin in the developing embryo also demands further study. Do these molecules participate in a hypothetical signalling system of intercellular communication functioning before the formation of specialised nervous and hormonal systems? We now have many sophisticated experimental tools (*in vitro* culture systems, ICSI, RT-PCR, microarrays) which will allow us to answer these and many other intriguing questions.

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Reference

AKASAKA, K., NASU, T., KATAYAMA, T. and MURAKAMI, N. (1995), Development of regulation of melatonin release in pineal cells in chick embryo. *Brain Res.* 692: 283-286.

BEKHTINA, V. (1966), Morfologicheskyie osobiennosti oligospermnogo oplodotworenija u kur. In: Nasledstwennost i izmienchivost' selskokhozyaistwiennoi ptitsy, Ed. Fomin A.I., Moscow, Izdatielstvo "Kolos". (in Russian). Morphologic particularities of oligospermic fertilisation in hen. In: Heritability and variability in the domestic fowl. Ed. Kolos, Moscow

BIRKHEAD, T.R., SHELDON, B.C. and FLETCHER, F. (1994), A comparative study of sperm-egg interactions in birds. *J. Repr. Fert*. 101: 353-361.

BRAMWELL, R.K. and HOWARTH, B., Jr.(1992), Preferential attachment of cock spermatozoa to the perivitelline layer directly over the germinal disc of the hen's ovum. *Biol. Repr*. 47: 1113-1117.

BUZNIKOV G.A., LAMBERT, H.W. and LAUDER, J.M. (2001), Serotonin and serotonin-like substances as regulators of early embryogenesis and morphogenesis. *Cell Tissue Res.*16: 177-186.

CALLEBAUT, M. (1968), [^{3}H]Uridine incorporation during previtellogenesis and early vitellogenesis in the oocytes of the chick (*Gallus gallus). J. Embryol. Exp. Morphol*. 20:169-174.

CALLEBAUT, M. (1973), Correlation between germinal vesicle and oocyte development in the adult Japanese quail (*Coturnix coturnix japonica)* A cytochemical and autoradiographic study. *J. Embryol Exp. Morphol*. 29: 145-157.

CALLEBAUT, M. (1974), La formation de l'oocyte d'oiseau: Etude autoradiographique chez la caille japoneuse (*Coturnix coturnix japonica*) pondeuse a l'aide de la leucine tritiee. *Arch. Biol*. (Bruxelles) 85: 201-233.

COS, S., GARCIA-BOLANDO, A. and SANCHEZ-BARCELO, E.J. (2001), Direct antiproliferative effects of melatonin od two metastatic cell sublines of mouse melanoma (B16BL6 and PG19). *Melanoma Res.* 11:197-201.

DANILOVA, N., KRUPNIK, V.N., SUDGEN, D. and ZHDANOVA I.V. (2004), Melatonin stimulates cell proliferation in zebrafish embryo and accelerates its development. *FASEB J.* 18: 751-753.

DAVIDSON, E.H., Gene Activity in Early Development (1986), Academic Press Ltd, London, pp. 670.

EMMANUELSSON, H., CARLBERG, M. and LOWKVIST, B. (1988), Presence of serotonin in early chick embryos. *Cell Differ.* 24: 191-200.

FUKUMOTO, T., KEMA, I.P. and LEVIN, M. (2005), Serotonin signalling is a very early step in patterning of the left-right axis in chick and frog embryos. *Curr. Biol.* 15:794-803.

GORBIK, T.A. (1976), On the ultrastucture and transformation of transosomes: the specific organelles of the avian ovary. *Arch. Anat. Histol. Embryol.* 70: 213-220.

HAMBURGER, V. and HAMILTON, H.L. (1992), A series of normal stages on the development of the chick embryo. *Dev. Dynamics* 195: 231-272.

HRABIA, T., TAKAGI, S., ONO, T. and SHIMADA, K. (2003), Fertilisation and development of quail oocytes after cytoplasmic sperm injection. *Biol. Repr*. 69:1651-1657.

ILKOVA, A.G., REHAK, P., VESELA, J., CIKOS, S., FABIAN, D., CZIKOVA, S. and KOPPEL, J. (2004), Serotonin localisation and its functional significance during mouse preimplantation embryo development. *Zygote* 12: 205-213.

IMAIZUMI-SCHERRER, T., RANGINI, Z., VESQUE, C., DUQUESNOY P. and SCHERRER, K. (1989), Presence of globin gene transcripts in chicken oocytes and of a partially processes globin RNA in early embryos. *Differentiation*, 41: 22-33.

ISHIZUKA, B., KURIBAYASHI, Y., MURAI, K., AMEMIYA, A. and ITOH, M.T.(2000), The effect of melatonin on in vitro fertilization and embryo development in mice. *J. Pineal Res.* 28:48-51.

IWAO Y. and ELINSON, R.P (1990), Control of sperm nuclear behaviour in physiologically polyspermic newt eggs: possible involvement of MPF. *Dev. Biol*. 142: 301-312.

IWAO, Y., SAKAMOTO, N., TAKAHARA, K., YAMASHITA, M. and NAGAHAMA, Y. (1993), The egg nucleus regulates the behaviour of sperm nuclei as well as cycling of MPF in physiologically polyspermic newt eggs. *Dev. Biol*. 160: 15-27.

JONES, M.P., MELAN M.A. and WITT-ENDERBY, P.-A. (2000), Melatonin decreases cell proliferation and transformation in a melatonin-receptor–dependent manner. *Cancer Lett.* 151:133-143.

JOVE, M., COBOS, P., TORRENTE, M., GILABERT, R. and PIERA, V. (1999), Embryonic development of pineal gland vesicles: A morphological and morphometric study in chick embryos. *Eur. J. Morph.* 37: 29-35.

KAWASHIMA, T., STEPINSKA, U., KUWANA, T., OLSZANSKA, B. (2008), Melatonin receptor genes (mel-1a, mel-1b, mel-1c) are differentially expressed in the avian germ line. *Mol. Reprod. Dev.* (in press).

KOCHAV, S., GINSBURG, M. and EYAL-GILADI, H. (1980), From cleavage to primitive streak formation: A complementary normal table and a new look at the first stages of the development of the chick. II. Microscopic study and cell population dynamics. *Dev. Biol*. 79: 296-308.

KVETNOY, J.M. (1999), Extrapineal melatonin: location and role within diffuse neuroendocrine system. *Histochem. J.* 31: 1-12.

LAUDER, J.M. (1993) Neurotransmitters as growth regulatory signals: role of receptors and second messengers. *Trends Neurosci.* 16: 233-239.

LAUDER, J.M., WILKI, M.B., WU, CH. and SINGH, S. (2000), Expression of 5-HT$_{2A,}$ 5-HT$_{2B,}$ and 5-HT$_{2C}$ receptors in the mouse embryo. *Int. J. Dev. Neurosci.* 18: 653-662.

MALEWSKA, A. and OLSZANSKA, B. (1999), Accumulation and localisation of maternal RNA in oocytes of Japanese quail. *Zygote*, 7: 51-59.

MANES, C. (1969), Nucleic acid synthesis in preimplantation rabbit embryos. Quantitative aspects, relationship to early morphogenesis and protein synthesis. *J. Exp. Zool*. 172: 303-310.

MORI, M., YAMASHITA, M., YOSHIKUNI, M., FUKADA S. and NAGAHAMA Y. (1991), Maturation-promoting factor and p34^{cdc2} kinase during oocyte maturation of the Japanese quail. *Dev. Biol.* 146: 246-249.

MOUDGAL, E.P., PANDA, J.N. and MOHAN, P. (1992), Serotonin in egg yolk and relation to cage density, stress and production status. *Ind. J. Animal Sci.62*: 147-148.

MOLLER, W. and MOLLER, G. (1990), Structural and functional differentiation of

the embryonic chick pineal organ in vivo and in vitro. *Cell Tissue Res.* 260: 337-342.

OBLAP, R. and OLSZANSKA, B. (2001), Expression of melatonin receptor transcripts (mel-1a, mel-1b and mel-1c) in Japanese quail oocytes and eggs. *Zygote,* 9: 237-244.

OBLAP, R. and OLSZANSKA, B. (2003), Presence and developmental regulation of serotonin N-acetyltransferase transcripts in oocytes and early quail embryos (*Coturnix coturnix japonica). Mol. Repr. Dev.* 65: 132-140.

OBLAP, R. and OLSZANSKA, B. (2004), Transition from embryonic to adult transcription pattern of serotonin N-acetyltransferase gene in avian pineal gland. *Mol. Repr. Dev.* 67: 145-153.

OLSZANSKA, B. and BORGUL, A. (1993), Maternal RNA content in oocytes of several mammalian and avian species. *J. Exp. Zool.* 265: 317-320.

OLSZANSKA, B., MALEWSKA, A. and STEPINSKA U. (1996), Maturation and ovulation of Japanese quail oocytes under *in vitro* conditions. *British Poultry Sci.* 37: 929-935.

OLSZANSKA, B., STEPINSKA, U. and PERRY, M. (2002), Development of embryos from in vitro ovulated and fertilized oocytes of the quail *(Coturnix coturnix japonica*). *J. Exp. Zool.* 292: 580-586.

OLSZANSKA, B., MAJEWSKI, P., LEWCZUK, B. and STEPINSKA, U. (2007), Melatonin and its synthesizing enzymes (AA-NAT and HIOMT) in avian eggs and early embryos. *J. Pineal Res.* 42:310-318.

PAULSON, J. and ROSENBERG, M.D. (1972), The function and transposition of lining bodies in developing avian oocytes. *J. Ultrastruct. Res.* 40: 25-43.

PERRY, M. (1988), A complete culture system for the chick embryo. *Nature* 331:70-72.

REITER, R.J., TAN, D-X., OSUNA, C. and GITTO, E. (2000), Actions of melatonin in reduction of oxidative stress. *J Biomed. Sci.* 7: 444-458.

REITER, R.J., TAN, D-X., SAINZ, R.M., MAYO, J.C. and LOPEZ-BURILLO, L. (2002), Melatonin: reduing the toxicity and increasing efficacy of drugs. *J. Pharm. Pharmacol.* 54: 1299-1321.

RENAUD, F., PARISI, E., CAPASSO, A. and PRISCO, P. (1983), On the role of serotonin and 5-methoxy-tryptamine in regulation of cell divisions in sea urchin. *Dev. Biol.* 98: 37-46.

SARI, Y. and ZHOU, F.C. (2003), Serotonin and its transporter on proliferation of fetal heart cells. *Int. J. Dev. Neurosci.* 21: 417-424.

SAUER, L.A., DAUCHY, R.T. and BLASK D.E. (2001) Polyunsaturated fatty acids, melatonin and cancer prevention. *Biochem. Pharmacol.* 61: 1455-1462.

SCHJEIDE, O.A., GALEY, F., GRELLERT, E.A., I-SAN LIN, R., DE VELLIS, J. and MEAD, J.F. (1970), Macromolecules in oocyte maturation. *Biol. Reprod. (Suppl)* 2:4-43.

SCHULTZ, G.A., (1975), Polyadenylic acid-containing RNA in unfertilized and fertilized eggs of the rabbit. *Dev. Biol.* 44: 270-277.

STEPINSKA, U. and OLSZANSKA, B. (1983), Cell multiplication and blastoderm development in relation to egg envelope formation during uterine development of quail (*Coturnix coturnix japonica*). *J. Exp. Zool.* 228: 505-510.

STEPINSKA, U., MALEWSKA, A. and OLSZANSKA, B. (1996a), RNase A activity in Japanese quail oocytes. *Zygote* 4: 219-227.

STEPINSKA, U. and OLSZANSKA, B. (1996b), Characteristics of poly(A)-degrading factor present in the avian oocytes and early embryos. *J. Exp. Zool.* 276: 191-29.

STEPINSKA, U. and OLSZANSKA, B. (2001), Detection of deoxyribonuclease I and II activities in Japanese quail oocytes. *Zygote* 9: 1-7.

STEPINSKA, U. and OLSZANSKA, B. (2003), DNase I and II present in avian oocytes: a possible involvement in sperm degradation at polyspermic fertilisation. *Zygote* 11: 35-42.

STEPINSKA, U. and BAKST, M.R. Fertilization. In: Reproductive Biology and Phylogeny of Aves. Ed. B.G.M. Jamieson, Science Publishers, Enfield, Hew Hampshire, USA; Plymouth, UK 2006, pp.553-587.

TAN, D-X., MANCHESTER, L.C., HARDELAND, L., LOPEZ-BURILLO, S., SAINZ R.M. and REITER R.J. (2003), Melatonin: a hormone, a tissue factor, an autocoid, a paracoid, and an antioxidant vitamin. *J. Pineal Res.* 34: 75-78.

TAYLOR, M.V., GUSSE, M., EVAN, G.I., DATHAN, N. and MECHALI, M. (1986), Xenopus myc proto-oncogene during development: expression as a stable maternal mRNA uncoupled from cell division. *EMBO J.* 5: 3563-3570.

WACLAWEK, M., FOISNER, R., NIMPF, J. and SCHNEIDER, J. (1998), The chicken homologue of zona pellucida protein-3 is synthesized by granulosa cells. *Biol. Repr.* 59: 1230-1239.

WEISS, E.R., MANESS, P. and LAUDER J.M. (1998), Why do neurotransmitters act like growth factors? *Perspec. Dev. Biol.* 5: 323-335.

YOSHIMURA, Y., OKAMOTO, T. and TAMURA, T. (1993), Ultrastructural changes of oocyte and follicular wall during oocyte maturation in the Japanese quail (*Coturnix coturnix japonica). J. Repr. Fert.* 97: 189-196.

ZAJCHOWSKI, L.D. and ETCHES R.J. (2000), Transgenic chickens: Past, Present and Future. *Avian Poultry Biol. Rev.* 11:63-80.

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