

ALVARO CASTELLANOS M. & CIA.
ABOGADOS
AGENTES DE LA PROPIEDAD INTELECTUAL
Carrera 9 No. 83-89
Bogotá-Colombia

65
64

SUPERINTENDENCIA Y COMERCIO
Radicación: 01011964 03962002 Folios: 114
Fecha (REC): 2001-06-05 15:33:14
Trámite: 032 PATENTES D 1 REGISTRO/ 444 VESPA-CTA
Dependencia: 2000 DIVISION DE NUEVAS CREACIONES

Señora

Jefe de la División de Nuevas Creaciones

Superintendencia de Industria y Comercio

Ministerio de Desarrollo Económico

E. S. D.

Ref.. Expediente No. 01:011.964.

Solicitud de Patente de Invención para:

ACTIVACION "IN OVO" DE UN HUEVO DE AVE EN SU CASCARA.

Solicitante: OVO BIOSCIENCES, INC.

Yo, MARGARITA CASTELLANOS ABONDANO, mayor de edad, identificada y domiciliada como aparece al pie de mi firma, en atención a su oficio No. 1075 notificado por Estado el 19 de Abril de 2001, atentamente me permito dar respuesta al mismo de la siguiente manera :

1. Presentación de los documentos faltantes :

- Presento las copias certificadas de los documentos de prioridad US No.60/182432 de Febrero 15 de 2000, US No.60/182969 de Febrero 16 de 2000 junto con la traducción de carátula y reivindicaciones.
- Presento copias auténticas de los documentos de cesión y poder debidamente legalizados por Apostilla, junto con su traducción oficial, en el que consta así mismo la existencia y representación de la sociedad solicitante. En dichos documentos de cesión y poder se indica el lugar de constitución de la sociedad solicitante, la cual se constituyó de conformidad con las leyes de Georgia, EE. UU. A, con oficinas en 1180 E. Broad Street Athens., Georgia, Estados Unidos de América.

■ Sustitución de poder a nombre de MARGARITA CASTELLANOS.

1

2. De acuerdo con lo anterior, se adjuntan los siguientes documentos :

- Documentos de cesión y poder debidamente legalizados
- Sustitución de poder a nombre de **MARGARITA CASTELLANOS.**
- Copias certificadas junto con su traducción.

Cumplido así con lo dispuesto, atentamente solicito a Ud. se continúe con el trámite correspondiente a esta solicitud de Patente de Invención.

Del Señor Jefe,

Margarita Castellanos
Margarita Castellanos Abondano
C.C. No. 39.779.654 de Usaquén
T.P.A. No. 70819 del C. Sup. de la Judicatura.
Carrera 9 No. 93 - 09 - Santa Fe de Bogotá
Teléfonos: 610 74 80 y 610 74 80

Santa Fe de Bogotá, D.C.,
MCA

5 JUN. 2001

ALVARO CASTELLANOS M. & CIA.
ABOGADOS
Carrera 9 No. 93-09
Apartado 6349
Bogotá - COLOMBIA

Señor
Jefe de la División de Nuevas Creaciones
Superintendencia de Industria y Comercio
Ministerio de Desarrollo Económico
E. S. D.

Re.: PODER conferido por la sociedad . OVO BIOSCIENCES, INC.

Yo, XIMENA CASTELLANOS ABONDANO, mayor de edad, identificada y domiciliada como aparece al pie de mi firma, atentamente manifiesto a Uds. que sustituyo en la Dra.: MARGARITA CASTELLANOS ABONDANO, el poder que me confirió la sociedad OVO BIOSCIENCES, INC. en Gainesville, Georgia - ESTADOS UNIDOS DE AMERICA el 22 de Marzo de 2.001. Debidamente legalizado por Apostilla bajo el No. I-033289 el día 4 de Abril de 2.001, con las mismas facultades que me fueron conferidas, para tramitar la solicitud de Patente de Invención, titulada : " ACTIVACION "IN OVO" DE UN HUEVO DE AVE EN SU CASCARA ".

Del Señor Jefe,

Acepto :


Ximena Castellanos Abondano
C.C. No. 39.773.330 de Usaquén
T.P.A. No. 58.238 de Del Cons. Sup. de la Judicatura
Carrera 9 No. 93-09 - Santafé de Bogotá
Teléfono: 6107420 - 6107480


Margarita Castellanos Abondano
C.C. No. 39.779.654 de Usaquén
T.P.A. No. 70819 del Cons. Sup. de la Judicatura
Carrera 9 No. 93-09 - Santafé de Bogotá
Teléfono: 6107420 - 6107480

Santafé de Bogotá,
XCA/zgt

**DILIGENCIA DE AUTENTICACION DE FIRMA
CON PRESENTACION PERSONAL**

(Art. 1ro. del decreto 22 92 de 1989)

Ante el Notario Cuarenta y uno (41) del Circuito de esta
ciudad compareció el Doctor Ximeno

Castellanos Abandonado

quien Exhibió la cédula de ciudadanía número 39933330

expedida en USA y la tarjeta profesional

número 58236 del C.S.J. con

el presente documento dirigido a Superintendente

y declaró que la firma que aparece en él es suya

FIRMA AUTOGRAFA DEL DECLARANTE

27 ABR 2001

C. R. F. de Bogotá D.C.

HORIZO LA INTERIOR

**DILIGENCIA DE AUTENTICACION DE FIRMA
CON PRESENTACION PERSONAL**

(Art. 1ro. del decreto 22 92 de 1989)

Ante el Notario Cuarenta y uno (41) del Circuito de esta
ciudad compareció el Doctor Margarita

Castellanos Abandonado

quien Exhibió la cédula de ciudadanía número 39933330

expedida en USA y la tarjeta profesional

número 70817 del C.M. con

el presente documento dirigido a Superintendente

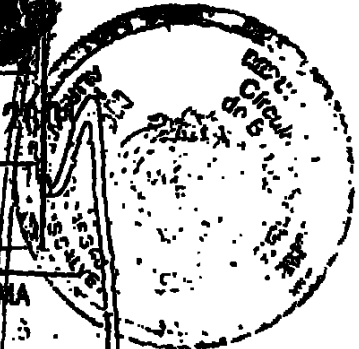
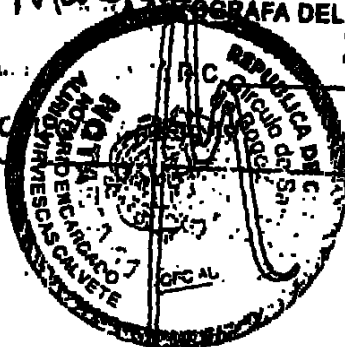
y declaró que la firma que aparece en él es suya

FIRMA AUTOGRAFA DEL DECLARANTE

27 ABR 2001

C. R. F. de Bogotá D.C.

AUTO



68

CERTIFICACION NOTARIAL
(Notarizing)

Yo, Notario Publico, CERTIFICO que el 22 de
Marzo del 2001

a quien(es) comparen y de quien(es) sea cuenta que de-
mos trece capacidad legal y que es(son) la(s) persona(s) que
firmo(ron) el documento anterior como
y en representación de

entidad legalmente constituida y que antes legítima-
mente ha sido la parte del Estado de
que conste en

Quilones ubique que dicho(s) persona(s) así(s) deli-
blemente facultado(s) para firmar el anterior documento
en nombre de la dicha entidad y que los mismos para
los cuales se otorga estas competencias dentro de las
fuerzas y atribuciones de tal entidad.

Lo establecido se hace en

de fecha
que se lee a la vista.

Day 22

Firmado en

NOTARIO PUBLICO

Notarizing

NOTARIAL CERTIFICATE
(Notarizing)

I, a Notary Public, CERTIFY that on 3-22-01
at Andrew Wooten & Timothy Cantrell

my newly appeared

to me known and known to me to be of legal capacity

and to be the person(s) who signed the foregoing in-
strument

and on behalf of OVO BioSciences, Inc.

a corporation duly organized and existing under the

laws of the State of Georgia

whose name shall be recorded in Athens

I further certify that said instrument(s) having been
read to said person(s) and that the purposes for which said
instrument is granted are within the scope of the
object or objects of said corporation.

The foregoing is based on Board Resolution

dated 3-01-2001

I signed

at Gainesville GA

Notary Public

CERTIFICACION NOTARIAL
(Notarizing)

Yo, Notario Publico, CERTIFICO que el
de 22

a quien(es) comparen y de quien(es) sea cuenta que de-
mos trece capacidad legal y que es(son) la(s) persona(s) que
firmo(ron) el documento anterior como
y en representación de

entidad legalmente constituida y que antes legítima-
mente ha sido la parte del Estado de
que conste en

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blemente facultado(s) para firmar el anterior documento
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los cuales se otorga estas competencias dentro de las
fuerzas y atribuciones de tal entidad.

Lo establecido se hace en

de fecha
que se lee a la vista.

Day 22

Firmado en

NOTARIO PUBLICO

NOTARIAL CERTIFICATE
(Notarizing)

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strument

and on behalf of OVO BioSciences, Inc.

a corporation duly organized and existing under the

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whose name shall be recorded in Athens GA

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dated 3-01-2001

I signed

at Gainesville GA

Notary Public

(Notarizing)

Yo, Notario Publico, CERTIFICO que el
22 de Marzo -2001

Andrew WOOTEN

a quien(es) comparen y de quien(es) sea cuenta que de-
mos trece capacidad legal y que es(son) la(s) persona(s) que
firmo(ron) el documento anterior como
y en representación de

Day 22

Firmado en Gainesville, GA.

NOTARIO PUBLICO

(Notarizing)

I, a Notary Public, CERTIFY that on 3-22-01

at Andrew Wooten & Timothy Cantrell

to me known and known to me to be of legal capacity

and to be the person(s) who signed the foregoing in-
strument

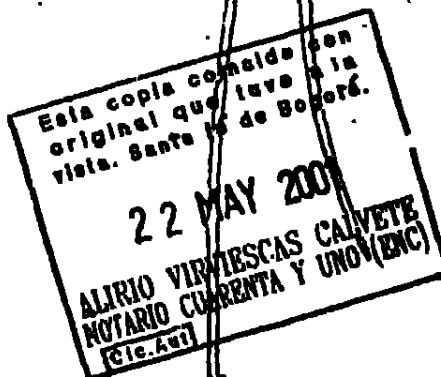
I signed

at Gainesville, GA

Notary Public

(NOTARIZING) Please read attached instructions to me in the margin

My commission expires
December 12, 2001.

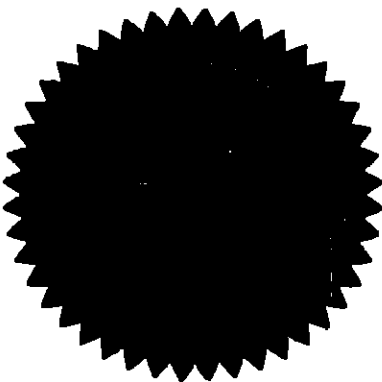


5

GEORGIA - HALL COUNTY.

I, ... DWIGHT S. WOOD Clerk of the Superior Court in
and for said County, the same being a Court of record, and being the Court under which the Notary
Public hereinafter named holds his appointment, do hereby certify that BROOKE
..... CAIN by and before whom the acknowledgement on the paper hereto attached
was taken was, at the time of taking the same a Notary Public in and for said County of Hall, regularly
commissioned and authorized by the laws of Georgia to attest deeds and take acknowledgements.

I further certify that I am familiar with the signature of the said BROOKE
..... HER
..... CAIN and that his signature to said paper appears to be genuine.
Witness my official signature and seal of said Court, This ...27TH.... day of MARCH....., 2001



Dwight S. Wood
.....
Clerk Superior Court, Hall County, Georgia

DORA DUQUE DE BERNAL
TRADUCTORA E INTERPRETE OFICIAL
C. 02759/08 Y 0114/97 - MINJUSTICIA
DEP. DE COLOMBIA

Esta copia coincide con
original que tuve a la
vista. Santa Fé de Bogotá.
22 MAY 2001
ALIRIO VIVIERSCAS CALVETE
NOTARIO CUARENTA Y UNO (ENC)
[Cic. Aut.]

4307

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24
20

**GEORGIA SUPERIOR COURT
CLERKS' COOPERATIVE AUTHORITY**

Notary Division

1875 Century Boulevard, Suite 100

Atlanta, Georgia 30345

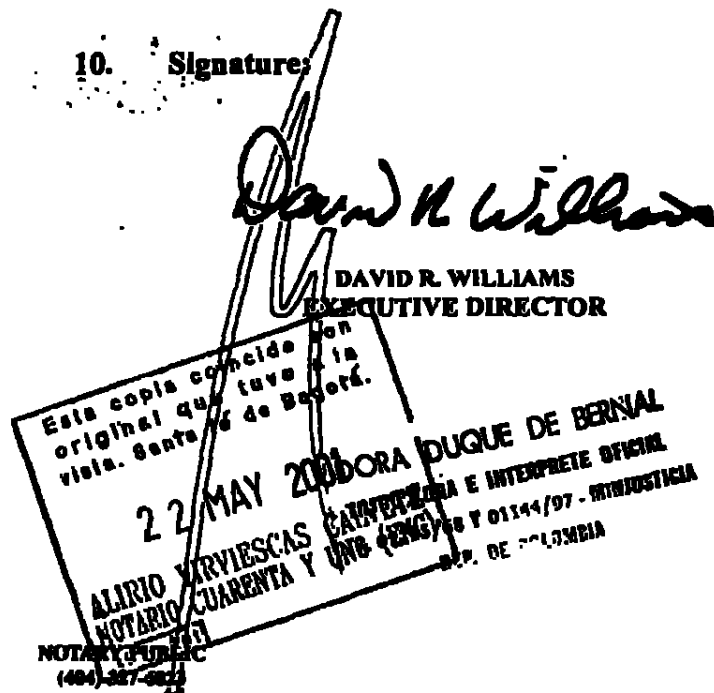
APOSTILLE

(Convention de La Haye de 5 Octobre 1961)

1. **Country: United States of America**
This public document
2. **has been signed by DWIGHT S WOOD**
3. **acting in the capacity of CLERK OF SUPERIOR COURT**
4. **bears the seal/stamp of SUPERIOR COURT, HALL COUNTY, GEORGIA**

CERTIFIED:

5. **at ATLANTA, GEORGIA**
6. **the 4TH DAY OF APRIL, 2001**
7. **by GEORGIA SUPERIOR COURT CLERKS' COOPERATIVE AUTHORITY**
8. **No. I-033289**
9. **Seal/Stamp:**
10. **Signature:**



71

_____**TRADUCCION OFICIAL No. 04-0420**_____

de un documento escrito en INGLES, el cual para su identificación lleva el sello de la Traductora e Intérprete Oficial, Res. No. 2755/88 y 0114/97 de Minjusticia, al igual que la presente.

(Anexos a un documento de cesión de Andrew Wooten y poder otorgado por Ovo Biosciences, Inc.)

GEORGIA – CONDADO DE HALL

Yo, DWIGHT S. WOOD, Escribano de la Corte Superior en y para dicho Condado, siendo la misma una corte de registro y la Corte bajo la cual el Notario Público antes mencionado aquí tiene su nombramiento, certifico por medio del presente que BROOKE CAIN por y ante quien fue tomado el reconocimiento en el documento anexo al presente, era en el momento de tomar el mismo un Notario Público en y para dicho Condado de Hall, normalmente comisionado y autorizado por las leyes de Georgia para certificar escrituras y tomar reconocimientos.

Certifico además que estoy familiarizado con la firma de dicho BROOKE CAIN y que su firma en dicho documento parece ser auténtica.

Da fe mi firma oficial y sello de dicha Corte, hoy día 27 de Marzo de 2001.

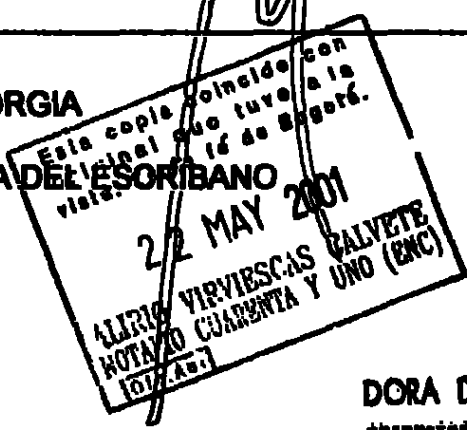
(fdo) Dwight S. Wood

Escribano de la Corte Superior, Condado de Hall, Georgia

(Sello en oblea dorada)

CORTE SUPERIOR DE GEORGIA

AUTORIDAD COOPERATIVA DEL ESCRIBANO



DORA DUQUE DE BERNAL
TRADUCTORA E INTERPRETE OFICIAL
RES. 2755/88 Y 0114/97 - MINJUSTICIA
REP. DE COLOMBIA

División Notarial

1875 Century Boulevard, Suite 100

Atlanta, Georgia 30345

APOSTILLA

(Convención de La Haya del 5 de octubre de 1961)

1. País: Estados Unidos de América

Este documento público

2. ha sido firmado por DWIGHT S. WOOD

3. actuando en su carácter de Escribano de la Corte Superior

4. lleva el sello de la Corte Superior, Condado de Hall, Georgia

Certificado

5. EN ATLANTA, GEORGIA

6. el día 4 de Abril, 2001

7. por la Autoridad Cooperativa de Escribanos de la Corte Superior

Georgia

8 No. I-033289

9. Sello

10. Firma:

(fdo) ilegible

David R. Williams

Director Ejecutivo

(Sello en oblea dorada)

ES TRADUCCION FIEL Y COMPLETA de cual me remito,

Bogotá, D. C., Abril 24, 2001



ORA DUQUE DE BERNAL
INTERPRETE OFICIAL
REG. 02755/00 Y 01144/97 - MINJUSTICIA
REP. DE COLOMBIA

MINISTERIO DE RELACIONES
EXTERIORES
Don Diego A. B.
12 433 b
CUNA FERIA APARECE EN EL
RECORDARIO DESEMPLEO
LAS FIRMAS INDICADAS

NO SE ASUME
RESPONSABILIDAD DEL TEXTO
2001 APR 26 P 12:24
FEE DE LEGALIZACION
ESTADO DE BOGOTÁ D.C.

ALVARO CASTELLANOS M. & CIA.
ABOGADOS
Carrera 9 No. 93-09
Apartado 6348
Bogotá - COLOMBIA

Señor
Jefe de la División de Nuevas Creaciones
Superintendencia de Industria y Comercio
Ministerio de Desarrollo Económico
E. S. D.

Re.: PODER conferido por la sociedad . OVO BIOSCIENCES, INC.

Yo, XIMENA CASTELLANOS ABONDANO, mayor de edad, identificada y domiciliada como aparece al plé de mi firma, atentamente manifiesto a Uds. que sustituyo en la Dra.: MARGARITA CASTELLANOS ABONDANO, el poder que me confirió la sociedad OVO BIOSCIENCES, INC. en Gainesville, Georgia - ESTADOS UNIDOS DE AMERICA en Marzo 22 de 2.001.

Debidamente legalizado por Apostilla bajo el No. I-033290 el día 4 de Abril de 2.001, con las mismas facultades que me fueron conferidas, para tramitar la solicitud de Patente de Invención titulada : " ACTIVACION "IN OVO" DE UN HUEVO DE AVE EN SU CASCARA ".

Del Señor Jefe,

Acepto :


Ximena Castellanos Abondano
C.C. No. 39.773.330 de Usaquén
T.P.A. No. 58.236 de Del Cons. Sup. de la Judicatura
Carrera 9 No. 93-09 - Santafé de Bogotá
Teléfono: 6107420 - 6107480


Margarita Castellanos Abondano
C.C. No. 39.778.654 de Usaquén
T.P.A. No. 70819 del Cons. Sup. de la Judicatura
Carrera 9 No. 93-09 - Santafé de Bogotá
Teléfono: 6107420 - 6107480

Santafé de Bogotá,
XCA/zgt

**DILIGENCIA DE AUTENTICACION DE FIRMA
CON PRESENTACION PERSONAL**

(Art. 1ro. del decreto 22 82 de 1989)

Ante el Notario Cuarenta y uno (41) del Circuito de esta
ciudad compareció el Doctor Ximena
Castellanos Abondano

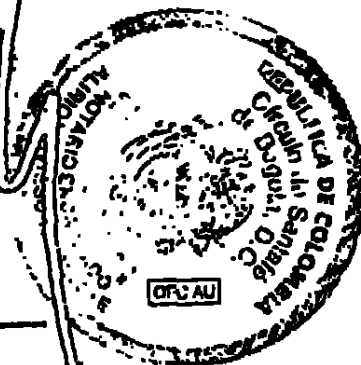
quien Exhibió la cédula de ciudadanía número 3977332
expedida en USA profesional
número 58736 con
el presente documento diligenciar Superintendencia
y declaró que la firma es suya.

Ximena Castellanos

FIRMA AUTOGRAFA DEL DECLARANTE

Santa Fé de Bogotá D.C. 27 ABR 2001

AUTORIZO LA ANTERIOR



**DILIGENCIA DE AUTENTICACION DE FIRMA
CON PRESENTACION PERSONAL**

(Art. 1ro. del decreto 22 82 de 1989)

Ante el Notario Cuarenta y uno (41) del Circuito de esta
ciudad compareció el Doctor Margarita
Castellanos Abondano

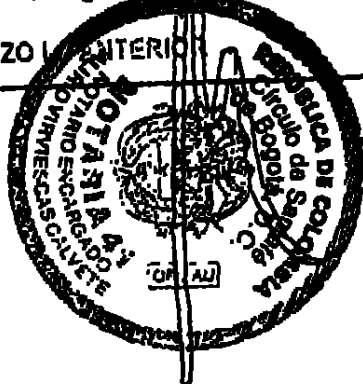
quien Exhibió la cédula de ciudadanía número 3977332
expedida en USA profesional
número 70112 con
el presente documento diligenciar Superintendencia
y declaró que la firma es suya.

Margarita Castellanos

FIRMA AUTOGRAFA DEL DECLARANTE

Santa Fé de Bogotá D.C. 27 ABR 2001

AUTORIZO LA ANTERIOR



12
2
75

Notarialis

CERTIFICACION NOTARIAL
(Notarialis)

Yo, Notario Publico, CERTIFICO que el **22 de Marzo del 2001** de la comparecencia ante mi

a quien(es) comparecieren y de quien(es) me consta que de su(s) capacidad legal y que comparecieren en su(s) propio(s) nombre(s) el documento anterior como y en representación de

personas debidamente constituidas y que están legítimas de todo las leyes del Estado de

Certifico además que dicho(s) person(s) no está(n) debidamente constituido(s) para firmar el anterior documento en nombre de la entidad señalada y que los mismos para los fines de este acto comparecieron dentro de las leyes y estatutos de tal entidad.

Lo testifico en la forma en

de fecho
que he testado a la vista.

Doy fe.

Firmado en

NOTARIO PUBLICO.

SPECIAL CERTIFICATE
(Comparation)

I, a Notary Public, CERTIFY that on **3-22-01**

(1) **Andrew Watson & Timothy Cantrell** before me personally appeared

to me known and known to me to be of legal capacity

(2) and to be the person(s) who signed the foregoing instrument

(3) and on behalf of **Ovo BioSciences, Inc**

a corporation duly organized and existing under the

(4) laws of the State of **Georgia**

(5) whose name shall be recorded in **Atlanta**

I further certify that said instrument(s) having been submitted to me for the foregoing instrument in the name of said corporation and that the purposes for which said instrument is granted are within the scope of the object or objects of said corporation.

(6) The foregoing is based on **Board Resolution**

(7) dated **3-01-2001**

(8) which have been

(9) signed by **Gainesville, GA**

Notary Public

CERTIFICACION NOTARIAL
(Notarialis)

Yo, Notario Publico, CERTIFICO que el **22 de Marzo del 2001** de la comparecencia ante mi

a quien(es) comparecieren y de quien(es) me consta que de su(s) capacidad legal y que comparecieren en su(s) propio(s) nombre(s) el documento anterior como y en representación de

personas debidamente constituidas y que están legítimas de todo las leyes del Estado de

Certifico además que dicho(s) person(s) no está(n) debidamente constituido(s) para firmar el anterior documento en nombre de la entidad señalada y que los mismos para los fines de este acto comparecieron dentro de las leyes y estatutos de tal entidad.

Lo testifico en la forma en

de fecho
que he testado a la vista.

Doy fe.

Firmado en

NOTARIO PUBLICO

SPECIAL CERTIFICATE
(Comparation)

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(1) **Andrew Watson & Timothy Cantrell** before me personally appeared

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(4) and on behalf of **Ovo BioSciences, Inc.**

a corporation duly organized and existing under the

(5) laws of the State of **Georgia**

(6) whose name shall be recorded in **Atlanta, GA**

I further certify that said instrument(s) having been submitted to me for the foregoing instrument in the name of said corporation and that the purposes for which said instrument is granted are within the scope of the object or objects of said corporation.

(7) The foregoing is based on **Board Resolution**

(8) dated **3-01-2001**

(9) which have been

(10) signed by **Gainesville, GA**

Notary Public

(Notarialis)

Yo, Notario Publico, CERTIFICO que el **22 de Marzo del 2001** de la comparecencia ante mi

a quien(es) comparecieren y de quien(es) me consta que de su(s) capacidad legal y que comparecieren en su(s) propio(s) nombre(s) el documento anterior como y en representación de **Timothy CANTRELL**

Doy fe.

Firmado en **Gainesville, GA.**

NOTARIO PUBLICO

(Comparation)

I, a Notary Public, CERTIFY that on **3-22-01**

(1) **Andrew Watson & Timothy Cantrell** before me personally appeared

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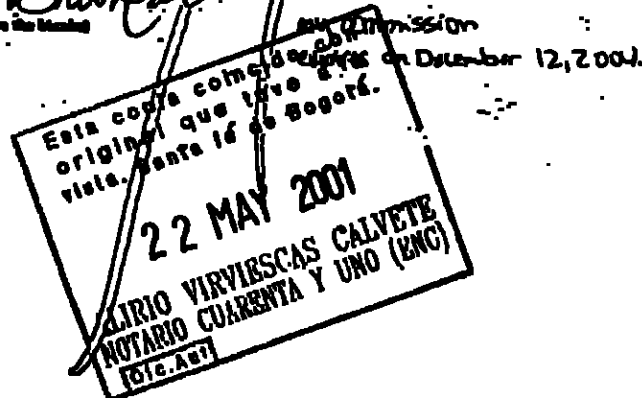
(9) dated **3-01-2001**

(10) which have been

(11) signed by **Gainesville, GA**

Notary Public

CERTIFICATION: These and attached instruments to be in the State



12

Columbiana 43
76

4307

**GEORGIA SUPERIOR COURT
CLERKS' COOPERATIVE AUTHORITY**

Notary Division

1875 Century Boulevard, Suite 100

Atlanta, Georgia 30345

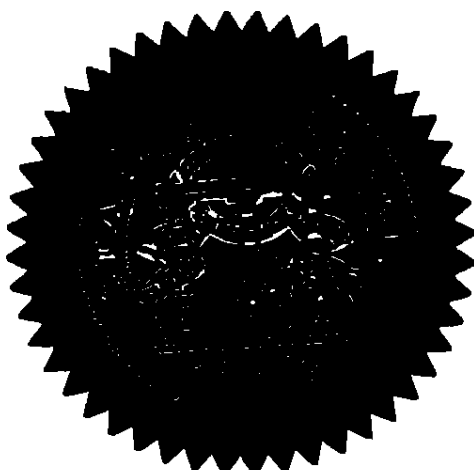
APOSTILLE

(Convention de La Haye de 5 Octobre 1961)

1. **Country: United States of America**
This public document
2. **has been signed by DWIGHT S WOOD**
3. **acting in the capacity of CLERK OF SUPERIOR COURT**
4. **bears the seal/stamp of SUPERIOR COURT, HALL COUNTY, GEORGIA**

CERTIFIED

5. **at ATLANTA, GEORGIA**
6. **the 4TH DAY OF APRIL, 2001**
7. **by GEORGIA SUPERIOR COURT CLERKS' COOPERATIVE AUTHORITY**
8. **No. I-033290**
9. **Seal/Stamp:**
10. **Signature:**



David R. Williams

**DAVID R. WILLIAMS
EXECUTIVE DIRECTOR**

DORA DUQUE DE BERNAL
TRADUCTORA E INTERPRETE OFICIAL
RES. 02755/68 Y 01144/97 - MINJUSTICIA
REP. DE COLOMBIA

NOTARY PUBLIC
(404) 317-4423

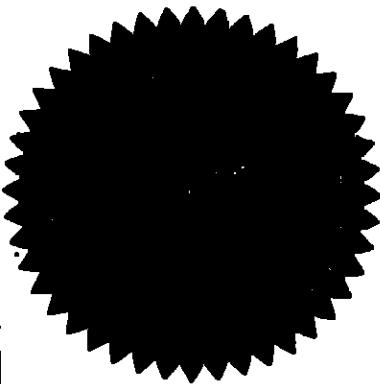


13

GEORGIA - HALL COUNTY.

I, ...DWIGHT S. WOOD..... Clerk of the Superior Court in
and for said County, the same being a Court of record, and being the Court under which the Notary
Public hereinafter named holds his appointment, do hereby certify that BROOKE.....
.....CAIN..... by and before whom the acknowledgement on the paper hereto attached
was taken was, at the time of taking the same a Notary Public in and for said County of Hall, regularly
commissioned and authorized by the laws of Georgia to attest deeds and take acknowledgements.

I further certify that I am familiar with the signature of the said BROOKE.....
HER
.....CAIN....., and that his signature to said paper appears to be genuine.
Witness my official signature and seal of said Court, This 27TH.. day of MARCH....., BK.....01



Dwight S Wood
.....
Clerk Superior Court, Hall County, Georgia

DORA DUQUE DE BERNAL
TRADUCTORA E INTERPRETE OFICIAL
RES. 02755/68 Y 01144/97 - MINJUSTICIA
REP. DE COLOMBIA

Esta copia coincide con
original que tuvo a la
vista. Santa Fe de Bogotá.
22 MAY 2001
ALDO VIRVIERSCAS CALVETE
NOTARIO CUARENTA Y UNO (ENC)
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_____**TRADUCCION OFICIAL N . 01-0421**_____

de un documento escrito en INGLÉS, el cual para su identificación lleva el sello de la Traductora e Intérprete Oficial, Res. No. 2755/68 y 0114/97 de Minjusticia, al igual que la presente._____

(Anexos a un documento de cesión de Timothy Cantrell y poder otorgado por Ovo Biosciences, Inc.)

GEORGIA – CONDADO DE HALL

Yo, DWIGHT S. WOOD, Escribano de la Corte Superior en y para dicho Condado, siendo la misma una corte de registro y la Corte bajo la cual el Notario Público antes mencionado aquí tiene su nombramiento, certifico por medio del presente que BROOKE CAIN por y ante quien fue tomado el reconocimiento en el documento anexo al presente, era en el momento de tomar el mismo un Notario Público en y para dicho Condado de Hall, normalmente comisionado y autorizado por las leyes de Georgia para certificar escrituras y tomar reconocimientos.

Certifico además que estoy familiarizado con la firma de dicho BROOKE CAIN y que su firma en dicho documento parece ser auténtica.

Da fe mi firma oficial y sello de dicha Corte, hoy día 27 de Marzo de 2001.

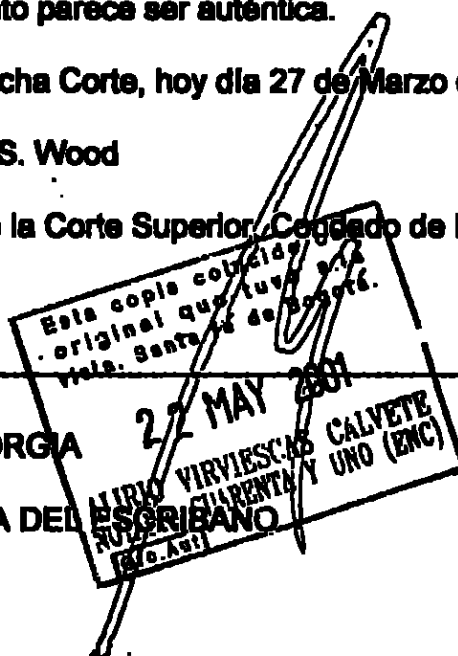
(fdo) Dwight S. Wood

Escribano de la Corte Superior, Condado de Hall, Georgia

(Sello en oblea dorada)

CORTE SUPERIOR DE GEORGIA

AUTORIDAD COOPERATIVA DEL



DORA DUQUE DE BERNAL
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REP. DE COLOMBIA

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División Notarial

1875 Century Boulevard, Suite 100

Atlanta, Georgia 30345

APOSTILLA

(Convención de La Haya del 5 de octubre de 1961)

1. País: Estados Unidos de América

Este documento público

2. ha sido firmado por DWIGHT S. WOOD

3. actuando en su carácter de Escribano de la Corte Superior

4. lleva el sello de la Corte Superior, Condado de Hall, Georgia

Certificado

5. EN ATLANTA, GEORGIA

6. el día 4 de Abril, 2001

7. por la Autoridad Cooperativa de Escribanos de la Corte Superior de Georgia

8 No. I-033290

9. Sello

10. Firma:

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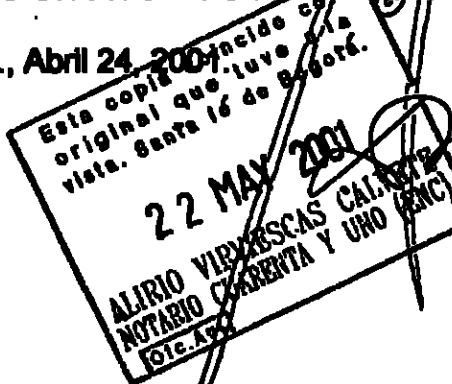
David R. Williams

Director Ejecutivo

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ES TRADUCCION FIEL Y COMPLETA de la cual me remito,

Bogotá, D. C., Abril 24, 2001



DORA DUQUE DE BERNAL

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RES. 02755/68 Y 01144/97 - MINJUSTICIA

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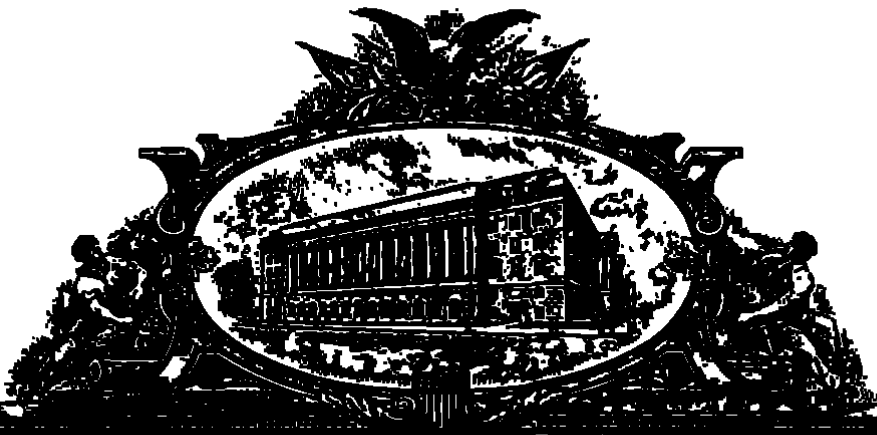
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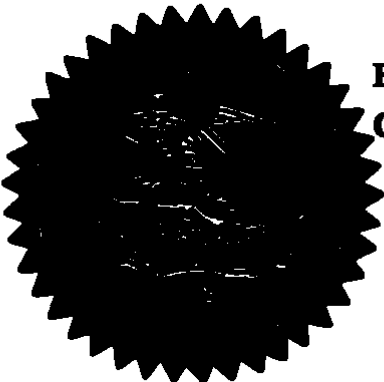
United States Patent and Trademark Office

March 28, 2001

THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A FILING DATE UNDER 35 USC 111.

APPLICATION NUMBER: 60/182,432

FILING DATE: February 15, 2000



**By Authority of the
COMMISSIONER OF PATENTS AND TRADEMARKS**

E. Bornett

**E. BORNETT
Certifying Officer**

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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

JCS20 U.S. PTO
02/15/00

JCS23 U.S. PTO
60/102436
02/15/00

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 C.F.R. § 1.53(c).

		Docket Number	23101.0001UI	Type a Plus Sign (+) inside this box - - - -	+
INVENTOR(s)/APPLICANT(s)					
LAST NAME	FIRST NAME	MIDDLE INITIAL	RESIDENCE (City and Either State or Foreign Country)		
Wooten Castrell	Andrew Timothy		1031 Scott Terrace, Atlanta, GA 30606 U.S. Citizen 3403 Maynard Cr., Gainesville, GA 30506-3735 U.S. Citizen		
TITLE OF INVENTION (280 characters max)					
METHOD OF FERTILIZING AN AVIAN EGG IN THE SHELL					
CORRESPONDENCE ADDRESS					
Len L. Kerber, Ph.D. NEEDLE & ROSENBERG, P.C. Suite 1200, The Candler Building 127 Peachtree Street, N.E. Atlanta					
STATE	Georgia	ZIP CODE	30303-1811	COUNTRY	U.S.A.
ENCLOSED APPLICATION PARTS (Check All That Apply)					
☒	Specification	Number of Pages	132 (including Claims and Abstract)		
☐	Drawing(s)	Number of Sheets	0		
		☐	Small Entity Statement		
		☐	Power of Attorney		
		☐	Other (specify)		

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METHOD PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT (Check One)			
☒	A check or money order is enclosed to cover the filing fees.		
☐	The Commissioner is hereby authorized to charge filing fees and credit Deposit Account Number:	FILING FEE AMOUNT	\$150.00
☒	The Commissioner is hereby authorized to charge any additional fees which may be required in connection with the following or credit any overpayment to Deposit Account No. 14-0629.		

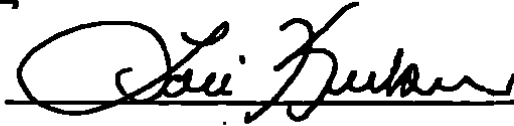
The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☐ No.

☐ Yes. The name of the U.S. Government agency and the Government contract number are:

Respectfully submitted,

SIGNATURE



Date

2/15/00

TYPED or PRINTED NAME: Lori L. Kerber

REGISTRATION NO. 41,113
(If Appropriate)

NEEDLE & ROSENBERG, P.C.
Suite 1200, The Candler Building
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I hereby certify that this correspondence is being deposited with the United States Postal Service as Express Mail Invoice No. EL273409627US in an envelope addressed to: BOX PROVISIONAL APPLICATION, Assistant Commissioner for Patents, Washington, D.C. 20231, on the date shown below.



Bruce Becker

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METHOD OF FERTILIZING AN AVIAN EGG IN THE SHELL

BACKGROUND OF THE INVENTION

5 FIELD OF THE INVENTION

The present invention relates to the field of avian egg fertilization. In particular, the present invention relates to a method of fertilizing an egg in a shell. The invention also relates to a method of fertilizing an egg in a shell, whereby a live chick is hatched. The invention also relates to an oviposited avian egg comprising a native embryo having
10 fewer than 40,000 cells, wherein the embryo can develop into a live chick.

BACKGROUND ART

It has long been a goal of avian geneticists to supplement traditional selection procedures by inserting desirable genes directly into the avian germline. Substantial
15 progress along these lines has been made in mammalian species where early embryos are accessible and where pronuclei can be visualized for the insertion of exogenous DNA. The production of transgenic mice, cattle, sheep, and goats has become a routine procedure in large commercial operations. In avian species, by contrast, the early embryo has not been easily accessed and the pronuclei are visually obscured, making
20 manipulation of the avian genome in early embryos *in ovo* an insurmountable goal.

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5 The difficulty involved in accessing an early stage avian embryo is best understood by understanding the anatomy of an avian egg and its progression through the female reproductive tract. At the time of ovulation, the avian oocyte comprises a blastodisc, or germinal disc, which contains the female pronucleus, and a yellow yolk mass. The germinal disc and yolk mass are surrounded by the oocyte cell membrane, called the oolemma. Surrounding the oolemma is the perivitelline layer (PL), also referred to as the inner perivitelline layer (IPL). The space between the oolemma and the IPL is termed the perivitelline space, which is traversed by granulosa cells. Once the oocyte is released from its ovarian follicle, it is referred to as an ovum. The ovum moves into the oviduct where it is engulfed by the infundibulum, where fertilization occurs if sperm are present.

15 As the ovum passes into the posterior infundibulum, another layer, the outer perivitelline layer (OPL), surrounds the ovum. This membrane acts to prevent polyspermy, which is a lethal condition that occurs when multiple sperm bind to and penetrate the ovum at the region of the blastodisc. The egg is then surrounded with additional layers of chalaza and thick and thin layers of albumen. When the ovum moves into the isthmus, two shell membranes are deposited, upon which small crystals of calcium carbonate are deposited, thus beginning the formation of the shell.

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The preceding events all occur within the first few hours following fertilization. The ovum next moves into the uterus, where over the next 18-20 hours, the calcium shell is completed. The egg then moves into the vagina for several minutes, and then is extruded from the vagina, or oviposited (i.e., "laid"). At this point, if the egg has been
5 fertilized, the embryo contained therein will have 40,000 to 70,000 cells. (Johnston ("In Vitro Sperm Binding, Penetration, and Fertilization of Recently Oviposited Chicken Eggs," December 1988, Clemson University); Olsen, M.W., J. Morph. 70: 413-533 (1942); Etches et al., in *Methods in Molecular Biology*, vol. 62 *Recombinant Gene Expression Protocols*, Ed. R. Tuan, Humana Press, Inc. Totowa, NJ, pp. 433-450
10 (1997); Petite et al., in *Manipulation of the Avian Genome*, Ed. Etches et al., CRC Press, Boca Raton, FL, pp.81-101 (1993)).

The development of genetically modified birds largely requires access to early stage, pluripotent embryonic cells. As the earliest stages of embryo development occur
15 within the oviduct of the female, the introduction of heterologous DNA into the early embryo has only been possible by removing the embryo from the female, requiring that she be sacrificed. Furthermore, while culture of the early stage embryo to hatch has been accomplished, the method is extremely laborious, and results in a post-hatch survival rate of only about 10%. Sang et al., in *Manipulation of the Avian Genome*, Ed.
20 Etches et al., CRC Press, Boca Raton, FL, pp.121-133 (1993).

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In addition, a common technique employed by the poultry industry is to artificially inseminate females. While this technique can allow for an increase in fertility and reduces the number of males required in the breeding program, it is extremely labor intensive.

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The present invention provides a ground-breaking improvement in avian biology by making it possible to fertilize an egg in its shell, and to hatch a live bird from the shell. The methods of the invention, therefore, make it possible to access an embryo as early as a zygote. The methods also allow for the automation of *in ovo* fertilization.

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SUMMARY OF THE INVENTION

The present invention provides a method of fertilizing an avian egg in a shell, wherein the egg comprises a yolk enclosed by a membrane, comprising obtaining a sperm sample comprising avian sperm in a physiologically acceptable carrier, and delivering the sperm sample into the egg, so as to fertilize the egg.

The present invention also provides a method of fertilizing an avian egg in a shell, wherein the egg comprises a yolk enclosed by a membrane, and hatching a live chick, comprising obtaining a sperm sample comprising avian sperm in a physiologically acceptable carrier, delivering the sperm sample into the egg, so as to fertilize the egg.

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incubating the egg, and hatching the live chick from the egg.

Also provided by the present invention is an oviposited avian egg comprising a native embryo having fewer than 40,000 cells, wherein the embryo can develop into a live chick. Relatedly, an oviposited avian egg comprising an embryo having fewer than 40,000 cells wherein the eggshell has an opening of less than 4 centimeters is also provided.

DETAILED DESCRIPTION OF THE INVENTION

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As used herein, "a," "an" or "the" may mean one or more. For example, "an" egg may mean one egg or more than one egg. Moreover, "the" egg may mean one egg or more than one egg.

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The present invention provides a method of fertilizing an avian egg in a shell, wherein the egg comprises a yolk enclosed by a membrane, comprising obtaining a sperm sample comprising avian sperm in a physiologically acceptable carrier, and delivering the sperm sample into the egg, so as to fertilize the egg. The present invention relates to the unexpected and surprising discovery that an unfertilized, oviposited avian egg can be fertilized in the shell. As used herein, reference to an avian egg in a shell refers to an oviposited egg, that is, an egg with a calcium carbonate shell that has been extruded from the vagina of the bird. Extrusion of the egg is referred to as

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"oviposition." Accordingly, all references herein to an "egg in a shell" or to an "oviposited egg" should be understood to be equivalent in meaning.

The process of fertilizing an oviposited egg in a shell is referred to herein as "in ovo fertilization" (IOF).

5

An avian egg comprises a hard, calcified shell at the time the egg is oviposited. Within the shell is a yolk that contains nutrients for supporting growth and development of an embryo. As used herein, an "embryo" is a developing organism resulting from the joining of a female pronucleus and a male pronucleus during the process of egg fertilization. While a fertilized (single cell) ovum may thus be called an embryo, the single cell embryo is also specifically referred to herein as a zygote.

10

Although in ovo fertilization can be performed on eggs as old as 2 weeks if the eggs are maintained at room temperature, ideally newly oviposited eggs are used for the best results. In a preferred embodiment, the sperm sample is delivered into the egg between 0 and 96 hours following oviposition. In a more preferred embodiment, the sperm sample is delivered into the egg between 0 and 72 hours following oviposition. In an even more preferred embodiment, the sperm sample is delivered into the egg between 0 and 48 hours following oviposition. In a highly preferred embodiment, the sperm sample is delivered into the egg between 0 and 24 hours following oviposition. Thus, it is preferred that the sperm sample be delivered into the egg as soon as possible

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following oviposition. However, the precise timing of fertilization can depend on how the oviposited egg is maintained, e.g., temperature, humidity, etc.

The sperm in the sperm sample may be obtained from a bird by methods known
5 to a person skilled in the art, such as the abdominal massage method which is well-known to those of skill in the art. This method allows the collection of an ejaculate comprising sperm, seminal fluid, and transparent fluid. Transparent fluid is a lymphlike fluid that passes from the lymph channels to the surface of the phallus during phallic
10 tumescence. Avian sperm may also be obtained from commercial sources that are well known to those of skill in the art.

In one embodiment, the sperm in the sperm sample can be collected from a single bird. In another embodiment, the sperm in the sperm sample is a mixture of sperm obtained from more than one bird. When a mixture of sperm from more than one bird is
15 used in the methods of the invention, the probability of successfully fertilizing the egg can increase, because if one of the birds from which the sperm has been collected is infertile, it is possible that the sperm collected from the other bird or birds will be capable of fertilizing the egg.

20 In a preferred embodiment, the sperm sample comprises sperm from birds which are members of the same species, and the sperm sample is used to fertilize eggs

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oviposited by hens which are members of the same species as the sperm donors. As one of ordinary skill is aware, individuals are classified as being from the same species if they are capable of mating to produce offspring. Moreover, the present invention also contemplates the use of sperm from one species and an egg from another species, if the sperm is capable of fertilizing the egg.

While it is typically preferred that the sperm be used within 30 minutes of the time that it is collected, older sperm, and even sperm which have previously been frozen, may be used in the methods of the invention, as long as the sperm retain their ability to fertilize an ovum. Where the sperm are to be used more than 30 minutes after collection, it is preferred that they be combined with a sperm extender, as is described below.

As mentioned above, the sperm sample also comprises a physiologically acceptable carrier. As used herein, a "physiologically acceptable carrier" is a fluid in which sperm remain motile and viable. Examples of a physiologically acceptable carrier include, but are not limited to, seminal fluid, transparent fluid, buffered saline solution, sperm extender, and combinations thereof. Preferably, the carrier will be a sperm extender, also referred to in the art as a diluent. As mentioned above, the use of a sperm extender is especially preferred where the collected sperm will not be used for fertilization within 30 minutes after collection. M.R. Bakst, In *Manipulation of the*

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Avian Genome, R. J. Etches and AM. Verrinder Gibbons, eds., CRC Press, Boca Raton, FL, pp. 15-28 (1993). As used herein, a "sperm extender" is a physiologically acceptable carrier that is used to dilute a sperm sample to produce a sperm sample of greater volume in which the sperm are less concentrated. Preferably, the composition of the sperm extender will extend the shelf life of the sperm, as well as diluting the sperm so as to increase the number of eggs which may be fertilized by the quantity of sperm which has been collected. Examples of sperm extender compositions, suggested dilution rates, optimal storage times and conditions, and commercial sources of extender may be found in Bakst ("Preservation of Avian Cells: *In: Poultry breeding and Genetics*, R.D. Crawford (ed.) Elsevier, New York, pp 91-108 (1990)). Another example of a sperm extender is the semen diluent described by Johnston ("In Vitro Sperm Binding, Penetration, and Fertilization of Recently Oviposited Chicken Eggs," December 1988, Clemson University). Other diluents commonly used in the poultry industry are Lago Formulation Avian Semen Extender by Hygeia Biological Laboratories, Semaide Turkey Extender by Poultry Health Laboratories in Davis California, Beltsville Poultry Semen Extender by Tri Bio Laboratories, Inc. in State College, Pennsylvania. In a preferred embodiment, the sperm extender is Avidiluent. Avidiluent is produced by IMB, 10 rue Georges, Clemenceau, BP 81, 61302 l'Aigle, France.

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Thus, in one embodiment, the sperm sample may comprise sperm and seminal

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fluid, i.e., semen. Moreover, the sperm sample may comprise sperm and seminal fluid which is diluted with a physiologically acceptable carrier, including but not limited to buffered saline solution and a sperm extender.

5 The sperm sample can also be prepared by methods which will be clear to one of ordinary skill in the art, such as washing semen from one or more birds with a solution such as buffered saline solution or sperm extender, centrifuging the resulting solution, removing the supernatant, and resuspending the washed sperm in a volume of a solution such as buffered saline or semen extender. One of ordinary skill in the art will readily
10 understand how to achieve the desired concentration of sperm by resuspending the sperm in the appropriate volume of solution. For example, following centrifugation and removal of supernatant, the packed sperm may then be weighed, and the number of sperm then estimated by using known values for the weight of avian sperm. The sperm may then be resuspended in the volume required to obtain the desired sperm
15 concentration. Alternatively, the centrifuged sperm may be resuspended following removal of the supernatant, and then recentrifuged, allowing the determination of the packed sperm volume. (Johnston, 1998). Subsequently, the concentration of the sperm may be calculated using the formula of Maeza and Buss. (Poultry Sci. 55:2059 (1976).)

20 Typically, the concentration of sperm in chicken semen is from 300 million to 800 million per milliliter, in turkey semen from 800 million to 1.5 billion per milliliter, in

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Guinea fowl semen from 400 million to 800 million per milliliter, in Pekin duck semen from 20 million to 600 million per milliliter. The standard number of sperm used for artificial insemination is 100 million in a total volume of 50 microliters. In the methods of the present invention, because sperm are placed directly adjacent to the female
5 pronucleus, far fewer sperm are required to fertilize the egg. Thus, as few as one sperm can be used in the methods of the present invention. In fact, a large range of sperm concentrations can be used in the present invention. In one embodiment, chicken semen is diluted with an equal volume of Avidihuent and approximately 0.01 milliliters of this sperm sample is injected into an egg. Thus, approximately 1 million sperm are deposited
10 adjacent to the female pronucleus.

In the methods of the invention, fertilization of the avian egg in the shell is accomplished by delivering the sperm sample into the egg. Delivery of the sperm sample may be accomplished by any method which will allow the sperm to be delivered inside
15 the shell, including, but not limited to, dissolving an area of the shell with, e.g., an acid solution, using electroporation, and creating an opening by penetrating or cracking an area of the shell, for example using a tool such as a needle or a scalpel.

Preferably, the surface of the area of the shell to be penetrated in order to deliver
20 the sperm sample is sanitized before the sperm is delivered inside, to prevent contamination of the egg. Any method which is compatible with the delivery method

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may be used to sanitize the shell, including, but not limited to, the disinfectant IOFEC-20®, and 3% hydrogen peroxide. The surface of the egg at the intended penetration site may be wiped or sprayed with the disinfectant, or the egg may be immersed in a vessel containing the disinfectant of choice.

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As is described above, an opening in the shell can be made with a tool such as a knife or a needle. Preferably, the tool will be sterile. For example, in a two-step procedure, an opening in the shell can first be made with a knife or other sharp instrument. In a second step, a needle attached to a syringe containing a sperm sample can be passed through the opening to deliver the sperm sample into the egg. Introduction of the sperm sample into the opening in the shell may also be accomplished by other means, including, but not limited to, the use of a pipette, such as a micropipettor. Alternatively, in one step, a needle attached to a syringe containing the sperm sample can be used to penetrate and thus create the opening in the shell and deliver by injection the sperm sample into the egg. Thus, "opening" can include a hole created by a needle. Of course, one of ordinary skill in the art will be able to choose a needle whose gauge will be large enough to allow the sperm sample to be moved through the needle. In one embodiment, the needle will be of the smallest gauge that can deliver intact sperm into the shell and also be sturdy enough to penetrate the calcium eggshell. Alternatively, a separate needle or other device could be used to make the opening in the eggshell. Typically, needles varying from 30-gauge to 16-gauge can be

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used. In one embodiment a 22-gauge needle is used. A sperm is approximately 0.5 um at its widest point and 100um in length. Therefore, any needle with an inner diameter of at least 10 um can be used for injections. In one embodiment the needle can remain in the shell after injection.

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The opening can be made anywhere in the shell that effects viable fertilization, but is preferably made in an area of the shell that is near the germinal disc. While an egg may be manipulated so as to place the germinal disc at different regions of the egg, the germinal disc in a newly oviposited egg is located at the large end of the shell, which
10 overlies the air cell adjacent to the yolk. Once an opening has been created in the shell, the sperm sample is preferable delivered by introducing the needle, pipette, etc., through the air cell and beyond a membrane lying below the air cell. In a further preferred embodiment, the sperm sample is delivered into the egg using a needle.

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Preferably, the needle, pipette, etc., is advanced through the shell at an angle of approximately 15°, penetrating the membrane lining the shell. In a preferred method of practicing the methods of the invention, the needle, pipette, etc., is advanced through the air cell, until it meets a membrane which encloses the yolk and germinal disc. A person practicing the method of the invention will know that the tip of the needle, pipette, etc.,
20 has encountered the membrane when slight resistance to further advancement of the tip is felt. As the tip is gently advanced, the resistance from the membrane gives way and

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the tip is allowed to barely penetrate the membrane. Preferably, the sperm sample is then delivered into the egg, adjacent to a region of the membrane and that is adjacent to the germinal disc. Typical volumes of the sperm sample are as small as 0.005 ml or as large as 0.10 ml. A preferred volume of injected sperm sample is about 0.01 ml.

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Preferably, to prevent contamination of the egg and death of an embryo, the opening in the shell is sealed. A non-toxic adhesive can be applied directly to the opening in the shell to seal it. Alternatively, a piece of eggshell can be used as a patch to close the opening and may be attached to the shell with a non-toxic adhesive. In one
10 embodiment, the non-toxic adhesive is Elmer's® glue. Moreover, any "tissue glue" can also be used to seal the shell. A "tissue glue" is a sterile, non-toxic adhesive used during surgical, operative procedures to bind tissues together.

The method of the present invention can be used to fertilize oviposited eggs from
15 avian species selected from the group consisting of chicken, quail, duck, turkey, pheasant, ostrich, emu, goose, peafowl, grouse, rhea, parrot, cockatiel, cockatoo, parakeets, and other commercially valuable birds.

The present invention also provides a method of fertilizing an avian egg in a
20 shell, wherein the egg comprises a yolk enclosed by a membrane, and hatching a live chick, comprising obtaining a sperm sample comprising avian sperm in a physiologically

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acceptable carrier, delivering the sperm sample into the egg, so as to fertilize the egg, incubating the egg, and hatching the live chick from the egg.

After the sperm sample has been delivered into the egg according to the
5 methods of the present invention as described above, the egg is incubated until the live chick is hatched. One of ordinary skill will be aware of the amount of time and the preferred conditions for incubating a fertilized egg belonging to a particular species. The following are incubation periods for various species of birds: Chicken -21 days, Quail-
23 days, Cornish quail- 17 to 18 days, Pheasant-23days, Turkey-28 days, Duck-28 to 33
10 days, Goose-28 to 30 days, Parakeet-18 days, Parrots-28 days, Dove-14 days, Mynah-14 days, Finch-14 days, Button Quail-16 days, Valley Quail-21 to 22 days, Swan-30 to 37 days. Incubation of eggs fertilized by the methods of the present invention as compared to naturally fertilized eggs need differ only in that the length of incubation time must be lengthened to include the amount of time that the fertilized egg would have
15 spent within the body of the female prior to oviposition. In a preferred embodiment, the incubation period lasts from 21 to 23 days for chicken eggs. While one of ordinary skill in the art will readily be able to determine the optimal temperature for incubation of an egg from a particular species of bird, typically the incubation temperature is between 95° F and 100° F. A chicken egg will be incubated at about 99.5° F. In a more preferred
20 embodiment, the temperature at which the chicken egg is incubated will be lowered as the egg nears the point of hatching. Thus, in a preferred embodiment, a chicken egg is

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incubated at 99.5° F from day 1 of incubation to about day 18 of incubation, and at 98.5° F from day 19 of incubation to hatching.

As is well known in the art, the humidity level at which an egg is incubated can be important for bringing the egg to hatch. Thus, typically the egg is incubated at between 75% and 90% humidity. Preferably, the egg is incubated at about 80% humidity. More preferably, the humidity level at which the egg is incubated will be raised as the egg nears the point of hatching. Thus, in a preferred embodiment, a chicken egg is incubated at 80% humidity from day 1 of incubation to about day 18 of incubation, and at 85% humidity from day 19 of incubation to hatching. In a specific preferred embodiment, an egg is incubated at 99.5° F and 80% humidity from day 1 of incubation to about day 18 of incubation, and at 98.5° F and 85% humidity from day 19 of incubation to hatching. As is well known in the art, turning the eggs during incubation is useful for promoting growth of the embryo.

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It is further preferred that the incubation of the eggs take place in a commercial incubator. Commercial hatchers and setters are produced by many companies including PAS Reform, Jamesway, Chickmaster, Buckeye, Cumberland, Petersime, Humidaire Incubator Co., etc. Preferably, the eggs are moved from a setter incubator to a hatcher incubator at about 3 days prior to hatch. The hatcher basket allows the egg to lie on its side where the chick can more easily pip out. This basket also allows the chick to walk

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about immediately after hatch, which is necessary for the chicks development and viability.

5 In another embodiment, the present invention provides an oviposited avian egg comprising a native embryo having fewer than 40,000 cells, wherein the embryo can develop into a live chick. "Native" means growing, living or produced in its place of origin. Thus, a native embryo is an embryo that develops and hatches the same shell in which the female pronucleus was formed. By the time an ovum which has been fertilized naturally has been oviposited, the developing embryo has between 40,000 and 10 70,000 cells. However, the egg of the present invention is fertilized after it has been oviposited in its shell; thus, an embryo developing in the egg of the present invention will at some time during incubation have fewer than 40,000 cells. In fact, at the moment of fertilization, the embryo in the egg of the present invention will have one cell and is a zygote. As the embryo grows within the egg, normal cell division will occur and the 15 number of cells will increase. Thus, the fertilized, oviposited egg of the present invention will at some time during incubation comprise an embryo having, for example, 10,000, 20,000 or 30,000 cells, including less than and between these numbers of cells.

20 In another embodiment, the present invention provides an avian egg in a shell comprising an embryo having fewer than 40,000 cells, wherein the embryo can develop into a live chick, and wherein the shell has an opening of less than 4 centimeters. In

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another embodiment, the opening in the shell is less than 2 centimeters. In another embodiment, the opening in the shell is less than 1 centimeter. In one embodiment, the opening in the shell is only large enough to accommodate a 22-gauge needle. The embryo can be either native or non-native to the egg.

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An egg of the present invention may, for example, be derived from avian species selected from the group consisting of chicken, quail, duck, turkey, pheasant, ostrich, emu, goose, peafowl, grouse, rhea, parrot, cockatiel, cockatoo, parakeets, swan, dove, and other commercially valuable birds. In a commercially preferred embodiment, the egg is derived from avian species used in the methods of the present invention and is selected from the group consisting of chicken, turkey, goose, duck, quail, and pheasant. In a more preferred commercial embodiment, the egg is derived from a chicken. The method can also be effectively utilized on avian species in zoos, e.g., to help preserve endangered species, including but not limited to those found in McGrew's zoo.

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The methods of the present invention can also be used for in ovo fertilization of reptilian eggs. Reptilian eggs, similar to avian eggs, comprise a yolk and female pronucleus and are protected by a shell when they are laid. An unfertilized, oviposited reptilian egg can be fertilized in the shell according to the methods of the present invention. In particular, a sperm sample, comprising sperm from one or more reptiles of the same species, is delivered into the unfertilized, oviposited egg through an opening

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created in the shell and onto the yolk adjacent to the female pronucleus where fertilization occurs.

The present invention also provides a method for producing an avian embryo
5 containing heterologous nucleic acid comprising, fertilizing an avian egg by the method
of the present invention disclosed herein, and introducing heterologous nucleic acid into
the avian egg. As used herein, nucleic acids include, but are not limited to, DNA and
RNA. The nucleic acids may be single, double, or multiple-stranded.

10 After an avian egg has been fertilized by IOF, the developing embryo can be
accessed at any stage of development to manipulate the genetic makeup of some or all
of its cells. Of greatest interest for creating transgenic birds is the early embryo. IOF
allows for germ line transmission of the heterologous nucleic acid as it allows the
earliest possible introduction of heterologous nucleic acid into the early avian genome.
15 This genetic manipulation of the developing embryo can, for example, produce
transgenic birds comprising genetic material which can be used to modulate endogenous
DNA and its expression and to create cells which can manufacture commercially
valuable proteins for commercial use.

20 Transfer of nucleic acid into the avian genome can be performed by a person
skilled in the art according to several known methods. (Harlow and Lane, *Antibodies*:

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- A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1988; Lennette et al., *Manual of Clinical Microbiology*, 14th Ed., Amer. Soc. for Microbiology, Washington, D.C., 1985; Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989; *Antisense RNA and DNA*, D. A. Melton, Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1988); Freshney, *Culture of Animal Cells, A Manual of Basic Technique*, 2nd Ed. Alan R. Liss, Inc., New York (1987); Centers for Disease Control Laboratory Manual, U.S. Department of Health, Education, and Welfare Pub. No. 79-8375, p. 75, Centers for Disease Control, Atlanta, GA;
- 10 *Fundamental Virology*, 2nd Ed. Bernard N. Fields and David M. Knipe, Chief Eds., Raven Press: New York, 1990). Examples of methods of transferring genetic material into the avian genome include, but are not limited to, avian leukosis virus (ALV) transduction mediated transgenesis, transposon mediated transgenesis, blastodermal cell mediated transgenesis, primordial germ cell mediated transgenesis and nuclear transfer.
- 15 The heterologous nucleic acid may be introduced into the embryo at the same time that the sperm sample is introduced into the egg, or at a later stage of embryonic development.

- A standard method now in use for producing transgenic birds is to produce
- 20 replication deficient ALV-derived transducing particles comprising a DNA insert. Because the particles are not capable of replicating in the bird, there is no risk of causing

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viremia and illness in the bird or humans. A window can be generated just above the embryo and transducing particles introduced into the subgerminal cavity of the embryo. The window is then sealed, and the eggs are placed into incubators for development.

5 Another method used to transfer heterologous nucleic acid into the avian genome is transposon-mediated transgenesis. Transposons are genetic elements that are able to translocate or move about within the genome of their host species. There are no known transposons that occur in avian species. However, it has been shown that certain transposons, reconstructed from other sources, are able to function within the avian genome. These transposons can be engineered to serve as useful nucleic acid vector systems for inserting genes into the avian genome.

Further, another method for introducing heterologous DNA into an avian embryo is blastodermal cell transgenesis. Blastodermal or other early cells can be taken from stage X embryos (the embryonic stage at which a fertilized egg is laid) can be injected back into recipient embryos where they colonize and grow, giving rise to chimeric chickens. A "chimeric" chicken is composed of cells from two different genetic lineages. Blastodermal cells injected into irradiated host embryos soon after they are isolated have the ability to contribute to all tissues of the resulting bird including the germline (cells that give rise to sperm and eggs and thus to all following generations).

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Blastodermal cells have been cultured and transfected with DNA by various methods known to persons skilled in the art in an attempt to generate transgenic poultry.

Thus, blastodermal cells or embryonic stem (ES) cells can be injected into an in
5 ovo fertilized embryo to create chimeric poultry. These chimeric poultry can then be crossed and, assuming they have germ line transmission of the blastodermal or ES cells, one can create a clone of the original injected cell.

Moreover, primordial germ cell mediated transgenesis may be used to create
10 transgenic birds. As the developing embryo grows, certain cells are committed to the germline to give rise to the sperm and eggs. These primordial germ cells (PGCs) migrate to the genital ridge of the developing gonad where they lie dormant until the bird reaches sexual maturity. One approach to avian transgenesis is to isolate these PGCs, genetically manipulate them and place them back into a developing embryo for
15 continued development. This technical approach is comparable to the blastodermal cell approach in both the extent to which it is developed and its potential applications. Primordial germ cells have been shown to contribute to the germline when injected into recipient embryos just as blastodermal cells have.

20 Nuclear transfer is another technique by which a person skilled in the art can produce a transgenic bird. Nuclear transfer involves the transfer of a nucleus from a

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donor cell (nucleoplast) into an enucleated cytoplasm (cytoplast) to form a reconstituted cell with the genetic makeup of the donor nucleus. Nuclear transfer has many scientific applications; however, the most widely publicized application is for the cloning of animals. Initially nuclear transfer was performed to derive clones from a single embryo to produce several identical cloned animals. Typically nuclei from an embryo at the 8 to 64-cell stage are introduced into enucleated oocytes to produce new single-cell embryos capable of development to form cloned animals. Clearly, the fertilization methods and fertilized avian eggs having less than 40,000 cells would make such work much easier, as the 8-64 cell embryo could easily be accessed without the necessity of sacrificing the female bird giving rise to the ovum. Nuclei from somatic cells such as fibroblasts and mammary epithelial cells can be combined with an enucleated oocyte where they are reprogrammed by cytoplasmic factors to become a new viable embryo capable of giving rise to a healthy animal.

The in ovo fertilization methods described herein can also be utilized in conjunction with other in ovo procedures. For example, the embryo can be vaccinated after fertilization. Alternatively, such vaccination could occur simultaneous with in ovo fertilization providing however that the vaccine did not prevent development of the embryo.

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Additionally, in ovo fertilization can be automated such that multiple eggs are simultaneously fertilized by, for example, injection techniques.

It will be clear to those of ordinary skill in the art that the methods of the present invention may easily be applied to a large-scale industrial operation, using automation to fertilize newly laid eggs. Accordingly, an apparatus which has previously been used, for example, to immunize newly fertilized eggs can be adapted to instead introduce a sperm sample.

The following Example is set forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how the methods claimed herein performed, and is intended to be purely exemplary of the invention and is not intended to limit the scope of what the inventors regard as their invention. Efforts have been made to ensure accuracy with respect to numbers (e.g., amounts, temperature, etc.) but some errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, temperature is in °F and pressure is at or near atmospheric.

EXAMPLE

1. Forty-three freshly laid unfertilized chicken eggs were disinfected by wiping the shells with 3% hydrogen peroxide and placed in racks.

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2. Sperm was obtained from 4 barred rock roosters on the same morning and collected in Vacutainer® vials less than 1 hour before the fertilization procedure was performed.
3. Sperm was pooled from the 4 roosters and mixed with 1 ml of Avidiluent®.
- 5 4. Sperm mixed with Avidiluent® was drawn into a 1 ml syringe through a 1", 22 gauge needle to form a sperm sample.
5. The needle created an opening in the large, blunt end of the eggshell and passed through the opening at a 15° angle to the surface of the shell.
6. The needle was passed through the air cell until the tip just penetrated the
10 membrane enclosing the yolk and germinal disc.
7. One drop, 0.05 ml, of the sperm sample was injected onto the surface of the yolk adjacent to the membrane.
8. The needle attached to the syringe was withdrawn from the egg.
9. The opening created in the shell by the needle was patched with a small piece of
15 shell, and the patch was secured to the shell with an adhesive such as Elmer's glue®.
10. The eggs were placed in commercial grade settlers maintained at 99.5°F and 80% humidity from day 1 to about day 18 of incubation. The eggs were turned according to methods known in the art and used in commercial settlers.
- 20 11. On day 19, the eggs were transferred to commercial hatchers and maintained at 98.5°F and 80% humidity until hatching.

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Ten days after the fertilization method of the present invention was performed on 43 eggs, routine candling of the eggs was performed to determine which eggs had been successfully fertilized. Thirty-five eggs of the 43 eggs had been
5 fertilized. Of the 35 fertilized eggs, 32 were successfully brought to hatching, and all but one of the chicks were healthy. Thus, 72% of the 43 oviposited eggs treated by the fertilization method of the present invention produced a healthy live chick.

10 Throughout this application, various publications are referenced. The disclosures of these publications, and the references cited therein, in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

15 It will be apparent to those skilled in the art that various modifications and variations can be made in the present invention without departing from the scope or spirit of the invention. Other embodiments of the invention will be apparent to those skilled in the art from consideration of the specification and practice of the invention disclosed herein. It is intended that the specification and example be considered as
20 exemplary only, with a true scope and spirit of the invention being indicated by the claims.

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What is claimed is:

1. A method of fertilizing an avian egg in a shell, wherein the egg comprises a yolk enclosed by a membrane, comprising:
 - a) obtaining a sperm sample comprising avian sperm in a physiologically acceptable carrier; and
 - b) delivering the sperm sample into the egg, so as to fertilize the egg.
2. The method of claim 1, wherein the egg and the sperm are derived from members of a same species.
3. The method of claim 1, wherein the carrier is diluted seminal fluid.
4. The method of claim 3, wherein the diluted seminal fluid comprises Avidiluent.
5. The method of claim 1, wherein the sperm sample is delivered adjacent to a membrane enclosing the yolk.
6. The method of claim 5, wherein the sperm sample is delivered adjacent to a region of the membrane that is adjacent to the germinal disc.

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7. The method of claim 2, wherein the avian species is selected from the group consisting of chicken, quail, duck, turkey, pheasant, ostrich, goose, and rhea.
8. The method of claim 7, wherein the avian species is chicken.
9. The method of claim 1, wherein the sperm sample comprises a mixture of sperm obtained from more than one bird.
10. The method of claim 1, wherein the delivery of the sperm sample is achieved by creating an opening in the shell by penetrating the shell and introducing the sperm sample into the egg.
11. The method of claim 10, wherein the opening is sealed.
12. The method of claim 11, wherein the opening is sealed with an adhesive.
13. A method of fertilizing an avian egg in a shell, wherein the egg comprises a yolk enclosed by a membrana, and hatching a live chick, comprising:
 - a) obtaining a sperm sample comprising avian sperm in a physiologically acceptable carrier;
 - b) delivering the sperm sample into the egg, so as to fertilize the egg;

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- c) incubating the egg of step b); and
- d) hatching the live chick from the egg.

14. The method of claim 13, wherein the egg and the sperm are derived from members of a same species.

15. The method of claim 13, wherein the carrier is diluted seminal fluid.

16. The method of claim 15, wherein the diluted seminal fluid comprises Avidihuent.

17. The method of claim 13, wherein the sperm sample is delivered adjacent to a membrane enclosing the yolk.

18. The method of claim 17, wherein the sperm sample is delivered adjacent to a region of the membrane that is adjacent to a germinal disc.

19. The method of claim 13, wherein the avian species is selected from the group consisting of chicken, quail, duck, turkey, pheasant, ostrich, goose, and rhea.

20. The method of claim 19, wherein the avian species is chicken.

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21. The method of claim 13, wherein the sperm sample comprises a mixture of sperm obtained from more than one bird.
22. The method of claim 13, wherein the delivery of the sperm sample is achieved by creating an opening in the shell by penetrating the shell and introducing the sperm sample into the egg.
23. The method of claim 22, wherein the opening is sealed.
24. The method of claim 23, wherein the opening is sealed with an adhesive.
25. The method of claim 13, wherein the egg is a chicken egg, and incubation of the egg lasts from 21 to 23 days.
26. The method of claim 25, wherein incubation is performed at a temperature of about 99.5°F from day 1 to about day 18 of incubation and a temperature of about 98.5°F from about day 19 of incubation to hatching.
27. The method of claim 25, wherein incubation is performed at 80% humidity from day 1 to hatching.

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28. The method of claim 13, further comprising vaccinating the embryo during step (c).
29. An oviposited avian egg comprising a native embryo having fewer than 40,000 cells, wherein the embryo can develop into a live chick.
30. The egg of claim 29, wherein the embryo has fewer than 30,000 cells.
31. The egg of claim 29, wherein the embryo is a zygote.
32. The egg of claim 29, further comprising a heterologous nucleic acid.
33. The egg of claim 29 which is a chicken egg.
34. An oviposited avian egg comprising an embryo having fewer than 40,000 cells, wherein the egg shell has an opening less than 4 centimeters.
35. The egg of claim 34, wherein the embryo has fewer than 30,000 cells.
36. The egg of claim 34, wherein the embryo is a zygote.

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37. The egg of claim 34, wherein the opening is sealed.
38. The egg of claim 34, wherein the opening is less than 1 centimeter.
39. The egg of claim 34, wherein the opening is less than 5 millimeters.
40. An egg produced by the method of claim 1, wherein the egg comprises a zygote.
41. A method for producing an avian embryo containing heterologous nucleic acid comprising:
- a) fertilizing an avian egg by the method of claim 1; and
 - b) introducing heterologous nucleic acid into the avian egg.
42. The method of claim 41, wherein the heterologous nucleic acid encodes a pharmaceutical protein, an antigen, a hormone or an antibody.
43. The method of claim 41, wherein the heterologous nucleic acid comprises Avian Leukemia Virus-derived transducing particles.

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Abstract of the Invention

The present invention relates to the field of avian egg fertilization. In particular, the present invention provides a method of fertilizing an egg in a shell. The invention
5 also provides a method of fertilizing an egg in a shell, whereby a live chick is hatched. The invention also provides an oviposited avian egg comprising a native embryo having fewer than 40,000 cells, wherein the embryo can develop into a live chick.

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DEPARTAMENTO DE COMERCIO DE LOS ESTADOS UNIDOS
Oficina de Patentes y Marcas de los Estados Unidos

Marzo 28, 2001

Por la presente se certifica que lo que obra anexo a ésta es copia fiel de los registros en la Oficina de Patentes y Marcas de los Estados Unidos de aquellos escritos de la solicitud de patente más adelante identificada que reunió los requisitos para que se le concediera una fecha de presentación sobre la base de 35 USC 111.

Número de Solicitud: 60/182,432

Fecha de Presentación: Febrero 15, 2000

**Por autoridad del
Comisionado de Patentes y Marcas**

**E. BORNETT
Funcionario certificante**

(Hay un sello-oblea)

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REIVINDICACIONES

1. Un método para fertilizar un huevo de ave en su cáscara, donde el huevo comprende un vitelo recubierto por una membrana y comprende:
 - a) obtener una muestra de esperma que comprenda un esperma de ave en un portador fisiológicamente aceptable y
 - b) administrar la muestra de esperma dentro del óvulo, para así fertilizar el huevo.
2. El método de la reivindicación 1, donde el huevo y el esperma se derivan de miembros de la misma especie..
3. El método de la reivindicación 1, donde el portador es un líquido seminal diluido.
4. El método de la reivindicación 1, en donde el líquido seminal diluido incluye Avidiluent.
5. El método de la reivindicación 1, en donde la muestra de esperma se administra en forma adyacente a una membrana que encierra el vitelo.
6. El método de la reivindicación 5, en el que la muestra de esperma se administra en forma adyacente a una membrana adyacente a un disco germinal.
7. El método de la reivindicación 2, en donde la especie de ave es seleccionada del grupo que consiste en gallinas, codornices, patos, pavos, faisanes, avestruces, gansos y ñandúes.
8. El método de la reivindicación 7, en donde la especie avícola es la gallina.
9. El método de la reivindicación 1, donde la muestra de esperma comprende una mezcla de esperma obtenido de más de un ave.

10. El método de la reivindicación 1, en donde la introducción de la muestra de esperma se efectúa practicando un orificio en la cáscara penetrando la misma e introduciendo la muestra de esperma en el huevo.
11. El método de la reivindicación 10, en el que el orificio es sellado.
- 5 12. El método de la reivindicación 11, en el que el orificio es sellado con un adhesivo.
13. Un método de fertilización un huevo puesto de ave dentro de su cáscara, en el que el huevo comprende un vitelo recubierto por una membrana, e incubar un pollo que comprende:
- 10 a) la obtención de una muestra de esperma que incluye esperma avícola en un vehículo fisiológicamente aceptable; y
- b) la administración de la muestra de esperma al interior del huevo, a fin de fertilizar el mismo.
- c) incubar el huevo de la etapa b) y
- 15 d) sacar del cascarón el pollo
- 14 El método de la reivindicación 13, donde el huevo y el esperma se derivan de miembros de la misma especie.
15. El método de la reivindicación 13, donde el vehículo es líquido seminal diluido.
- 20 16. El método de la reivindicación 15, donde el fluido seminal diluido comprende Avidiluent.
17. El método de la reivindicación 13, en el que la muestra de esperma es administrada en forma adyacente a una membrana que encierra el vitelo.
18. El método de la reivindicación 17, en el que la muestra de esperma es
- 25 administrada en forma adyacente a un sector de la membrana que es adyacente al disco germinal.

19. El método de la reivindicación 13, en el que la especie de ave es seleccionada del grupo que consiste en gallinas, codornices, patos, pavos, faisanes, avestruces, gansos y ñandúes.
20. El método de la reivindicación 19, en el que la especie avícola es la gallina.
21. El método de la reivindicación 13, en el que la muestra de esperma comprende una mezcla de esperma obtenido de más de un ave.
22. El método de la reivindicación 13, en el que la introducción de la muestra de esperma se efectúa practicando un orificio en la cáscara penetrando la misma e introduciendo la muestra de esperma en el huevo.
23. El método de la reivindicación 22, en el que el orificio es sellado.
24. El método de la reivindicación 23, en el que el orificio es sellado con un adhesivo.
25. El método de la reivindicación 13, donde el huevo es un huevo de gallina, y la incubación del huevo dura de 21 a 23 días.
26. El método de la reivindicación 25, donde la incubación se lleva a cabo a una temperatura de 99.5°F desde el primer día de incubación hasta el día 18 y una temperatura alrededor de 98.5°F desde el día 19 de incubación hasta el nacimiento.
27. El método de la reivindicación 25, donde la incubación se lleva a cabo a una humedad de 80% desde el primer día hasta el nacimiento.
28. El método de la reivindicación 13, que adicionalmente comprende vacunación del embrión durante el paso (C).
29. Un huevo de ave puesto que comprende un embrión nativo con menos de 40.000 células, en el que el embrión puede convertirse en un polluelo.

30. El huevo de la reivindicación 29, en el que el embrión presenta menos de 30.000 células.
31. El huevo de la reivindicación 29, donde el embrión es un cigoto.
32. El huevo de la reivindicación 29, que además comprende un ácido nucleico heterólogo.
33. El huevo de la reivindicación 29, el cual es un huevo de gallina.
34. Un huevo de ave puesto que comprende un embrión que tiene menos de 40000 células, donde la cáscara del huevo tiene un orificio de menos de 4 centímetros.
35. El huevo de la reivindicación 34, donde el embrión tiene menos de 30,000 células.
36. El huevo de la reivindicación 34, donde el embrión es un cigoto.
37. El huevo de la reivindicación 34, donde el orificio está sellado.
38. El huevo de la reivindicación 34, donde el orificio es menos de 1 centímetro.
39. El huevo de la reivindicación 34, donde el orificio es menos de 5 milímetros.
40. Un huevo producido por el método de la reivindicación 1, donde el huevo comprende un cigoto.
41. Un método para producir un embrión de ave que contenga ácido nucleico heterólogo que comprende:
- a) fertilizar un huevo de ave por el método de la reivindicación 1; y
 - b) introducir el ácido nucleico heterólogo dentro de un huevo de ave.
42. El método de la reivindicación 41, en el que el ácido nucleico heterólogo codifica una proteína farmacéutica, un antígeno, una hormona o un anticuerpo.

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43. El método de la reivindicación 41, en el que el ácido nucleico heterólogo incluye el Avian Leukemia Virus derivado de partículas transducing.

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March 27, 2001

THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A FILING DATE UNDER 35 USC 111.

APPLICATION NUMBER: 60/182,969

FILING DATE: February 16, 2000



**By Authority of the
COMMISSIONER OF PATENTS AND TRADEMARKS**

W. Montgomery
W. MONTGOMERY
Certifying Officer

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PROVISIONAL APPLICATION FOR PATENT
COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 C.F.R. § 1.53(c).

Docket Number		23101.0301U1		Type a Plus Sign (+) inside this box - - - -		+	
INVENTOR(s)/APPLICANT(s)							
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AUTOMATED SYSTEM FOR THE FERTILIZATION AND GENETIC ANALYSIS OF AVIAN EGGS							
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Respectfully submitted,

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Patent

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**PROVISIONAL APPLICATION
FOR
UNITED STATES LETTERS PATENT**

ON

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**AUTOMATED SYSTEM FOR THE FERTILIZATION AND
GENETIC ANALYSIS OF AVIAN EGGS**

BY

20

**Andrew L. Wooten, residing at 1031 Scott Terrace, Athens, Georgia 30606 and
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3735, both citizens of the United States of America.**

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BACKGROUND OF THE INVENTION

[illegible]

10 Background Art

Both natural breeding and artificial insemination are practiced at various levels within this hierarchy depending on the particular segment in question. Geneticists at the top of the breeding pyramid in broiler, layer, and turkey segments prefer artificial insemination to track pedigreed matings. Thus far, the layer segment has derived all subsequent generations in the pyramid from natural matings due to high reproductive efficiency in layers. By contrast, turkeys have been selected for broad breast size and are now unable to perform efficient natural matings. Thus, it becomes necessary to perform artificial insemination for the breeding of turkeys at all levels of the breeding pyramid.

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current practice is to restrict the diet of today's broiler breeders. Even so, the large size of the broiler breeder has compromised fertility and mating ability and it is predicted that fertility will continue to decline as body size continues to increase.

5 Several factors have limited the application of artificial insemination in poultry breeding schemes, particularly in broiler breeders. First, there is the high level of sophistication that is required in farm operations and its attendant cost. Another limitation has been the rapid deterioration of the fertilizing ability of semen stored in either liquid or frozen states. Accordingly, to date, artificial insemination has not been
10 practical on a large scale. The present invention overcomes these limitations existing in the art.

 As one skilled in the art appreciates, automated egg handling technologies have been developed simultaneously by many independent inventors worldwide. The
15 technologies existing today involve high-throughput systems for injecting liquid substances into avian embryonated eggs. Examples include the injection of vaccines to improve the immune state of resulting chicks, injection of viruses for the production of human and animal vaccines, and injection of proteins to influence chick health, growth, and the like. Another useful automated system for poultry production allows detection
20 of live versus dead chick embryos based on either candling with various light sources or temperature differentials between neighboring eggs in an egg flat. These technologies, taken together, represent the current state of development in this field. However, there exists a need for a more advanced platform adapted specifically to the needs of avian breeders, a need that the present invention addresses and satisfies.

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DETAILED DESCRIPTION OF THE INVENTION

 The present invention is more particularly described in the following disclosure that is intended as illustrative only since numerous modifications and variations therein
30 will be apparent to those skilled in the art. As used herein, "a," "an," and "the" can

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mean one or more, depending upon the context in which it is used. The presently preferred embodiment is now described.

Recent progress in the area of sperm preservation, taken together with the advent of In Ovo Fertilization technology, have made it possible to move towards a much more efficient management model for poultry breeding operations. Several of the important genetic technologies described below will be linked together to create a truly versatile platform for the automation of poultry breeding technologies.

10 The present invention incorporates a new technology called "In Ovo Fertilization (IOF)," which is addressed in a provisional patent application attached hereto as an Appendix. The IOF technique allows for avian oocytes to be fertilized inside the egg shell after the egg has been laid. This requires the injection of various formulations of avian sperm into the unfertilized egg. In order to perform this
15 procedure in an automated fashion, it is contemplated using two steps. The first step involves calculating the number of sperm cells in the sperm sample that is loaded onto the machine. This task can be accomplished through the use of an integrated spectrophotometer unit (sometimes called a densimeter) similar to those marketed by Animal Reproduction Systems of Chino California and others. The information
20 obtained from this analysis is communicated to a central processing unit or other analyzing system, in which optimal volumes of sperm and semen extender (diluent) are determined. This information is then communicated to a fluid dispensing mechanism, resulting in the correct fluid volumes to be dispensed into a central fluid reservoir of an egg injection mechanism or similar system.

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For the second step, the central processing unit activates the egg injection mechanism, which delivers the proper amount of the diluted sperm sample formulation into the unfertilized egg to the specific depth and at the appropriate angle to accomplish IOF as described in the Appendix attached hereto. The process of injection includes
30 perforation of the egg shell by a tubular punch and insertion of an injection needle through the vitelline membrane for delivery of the diluted sperm sample formulation.

The egg injection mechanism may be of a design similar to those manufactured and sold by Embrex, Inc., Merck Inc., and others in the industry. As an example, one design is disclosed in U.S. Patent No. 4,903,635, entitled "High Speed Automation Injection System for Avian Embryos," which is incorporated herein by reference. As described in the patent, the disclosed device is a high-speed automated injection system for avian embryos, which can inject eggs with fluid substances, specifically an inoculating fluid. The machine includes suction devices which lift eggs out of engagement with surfaces, rather than pushing them, before injecting them. Thus, the machine provides separate mechanisms and devices for first forming an opening in the egg shell and then injecting the avian embryo or the surrounding environment with a fluid substance, avoiding use of a single needle or punch to both puncture the shell of an egg and deliver fluid substances to the interior of the egg. As is also known in the art, the present invention here also contemplates using a single needle both to puncture the shell and to deliver fluid substances. Other relevant patents that disclose injection of fluids into eggs include U.S. Patent Nos. 5,900,929, entitled "Method and Apparatus for Selectively Injecting Poultry Eggs"; 5,722,342, entitled "Ovo Antibiotic and Microbial Treatment to Diminish Salmonellae Populations in Avians"; 5,699,751, entitled "Method and Apparatus for in Ovo Injection"; 5,438,954, entitled "Method and Apparatus for Early Embryonic in Ovo Injection"; 5,339,766, "Method of Introducing Material into Eggs During Early Embryonic Development"; 5,176,101, "Modular Injection System for Avian Embryos"; 5,158,038, "Egg Injection Method, Apparatus and Carrier Solution for Improving Hatchability and Disease"; and 5,136,979, "Modular injection system for avian embryos," all of which are incorporated by reference.

The process of IOF makes it possible to design systems capable of high-throughput operation for the fertilization of avian eggs after they reach the hatchery. Advantageously, the present invention requires much smaller quantities of sperm for this direct fertilization approach, making it possible to streamline operations by reducing dramatically the number of males in the breeding scheme. The remaining males could be centrally housed with enough fresh sperm being delivered directly to the hatchery to fertilize the billions of eggs hatched in the industry every year. The use

of fewer males would allow breeders to make more rapid genetic progress in improving their lines by using only the very elite performers for inseminations. In addition, the industry's infrastructure could be re-rationalized based on the elimination or significant reduction of males. For instance, a much more efficient commercial egg laying operation could be directly substituted for the existing hatching egg facilities since only unfertilized eggs are required. In artificial insemination programs where females represent from approximately ninety-five to ninety-eight percent (95-98%) of the flock, it will be possible to eliminate the need for manual insemination, and thus remove approximately over ninety-five percent (95%) of the current labor requirement associated with these programs.

In addition to fertilization, it is contemplated that the present invention also includes the processes of genetic analysis, manipulation, and propagation. As one skilled in the art appreciates, these tools are currently designed for laboratory usage requiring highly-skilled technicians and, accordingly, have been impractical to date for routine usage in the low-margin poultry industry. As implemented in the present invention, many of these technologies are amenable to incorporation into a totally automated platform for use by production personnel, as well as geneticists and other researchers.

The present invention can be designed to include additional mechanisms and steps to deliver various liquid formulations intended to (a) impact gene expression in the avian embryo concurrently with and/or subsequent to In Ovo Fertilization and/or (b) to provide vital information as to the genotypic status of the embryo. The delivery apparatus may be similar to the egg injection mechanisms for sperm delivery discussed above. The present invention may employ a common fluid reservoir with the sperm or a separate reservoir independent of the sperm, in which separate needles are inserted into the egg for each fluid, or the fluids are injected sequentially through a single needle inserted into the egg.

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The liquid formulations may include nucleic acids and/or nucleic acid vectors to be used to transfect the avian genome for the production of transgenic poultry. IOF, as described in the Appendix, allows one to obtain access to single-celled avian oocytes and early embryos.

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In contrast to the present invention, current systems allow access only to later stages of embryonic development. For instance, the typical chicken embryo is at the 60,000-cell stage when the egg is laid. Transformation of the avian embryo with exogenous DNA at this and later stages generates mosaic animals expressing the desirable product only in a small portion of their cells.

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Use of the present invention incorporates nucleic acids into single-celled oocytes and early embryos allows for the incorporation of exogenous DNA into all or most of the cells of the resulting bird. However, in an alternative embodiment, the present invention may be adapted to deliver these nucleic acids and nucleic acid vectors to later stage embryos. One current protocol for the production of transgenic poultry involves the injection of viral particles into the sub-germinal cavity of developing avian embryos. Another application would be for the transient expression of a particular gene product in the avian embryo. Nucleic acid formulations could be delivered with the specific intent of generating egg and/or animal based bioreactor systems. Proteins, antigens and antibodies could also be delivered to the embryo in order to affect gene expression.

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In another aspect of the present invention, liquid formulations can also include cells and/or cellular nuclei in suspension to be used in the generation of chimeric animals and/or cloned animals. Cells, such as avian blastodermal cells, have been injected into avian embryos for the express purpose of generating chimeric birds. This method has many research applications, such as the generation of transgenic animals, but could also find applications in production operations.

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A separate contemplated embodiment of the present invention involves the delivery of fluid suspensions containing cellular nuclei or "nucleoplasts." Nucleoplasts can be generated on a large scale using certain advanced centrifugation procedures. In many species such as sheep, cow, and mice it has been determined that a nucleoplast isolated from one cell can be inserted into another enucleated cell for the purpose of generating an identical cloned individual. If this procedure can be performed on an industrial scale, it would be very attractive to the poultry industry.

Although cloning or nuclear transfer has yet to be achieved in a poultry species, it is realistic to assume that it is will be only a short time until this goal is achieved. At that time, a high-throughput industrial platform will be needed. A separate mechanism such as a flow cytometric detector will probably be necessary in this embodiment to regulate the number of nucleoplasts (ideally one) that is delivered from the fluid reservoir to the avian egg.

Still another aspect of the present invention involves delivering liquid formulations to the avian egg that provide vital information about the genotypic status or constitution of the individual bird. For instance, DNA and/or RNA probes could be delivered into the avian egg for the purpose of sorting embryos by sex or any other genotype. These probes bind specifically to their target sequences and provide specific information about the genetic makeup of the bird. Labeling the probes with various fluorescent, radioactive, or chemiluminescent molecules provides highly-reliable technology for determining genotypic status. Alternatively, antibodies or other proteins may be utilized in a similar fashion as reporter molecules.

The present invention is also contemplated to incorporate a detection mechanism to aid in various types of genetic and protein based analyses. One embodiment of this mechanism would involve the use of a light source of the appropriate wavelength, such as, for example, a laser, and a corresponding dye set differentially expressed in the avian egg as an indicator of certain genotypic or physiologic states. For instance, this embodiment could sort eggs by sex utilizing

fluorescence-labeled sexing probes, sex-linked promoters and expression systems, fluorescence-labeled sexing antibodies, and the like.

5 The detection mechanism may, for example, use a CCD camera or other suitable detector of fluorescent signals to activate a sorting and/or identifying mechanism. In another embodiment, the detection mechanism could utilize a scintillation counter for radioaction and/or chemiluminescence-based detection methods for the same general purposes described above. In yet another embodiment, the system could utilize "gene-chip," genetic microarray and/or genetic macroarray
10 technologies for detection purposes, an example of which is that produced by the company Affymetrix.

As one skilled in the art appreciates, classification of birds according to genotype may be used in production operations. Classification of birds by sex allows
15 the optimization of production capacity. That is, males are desired in the broiler industry, while females are desired as layers. Also, the present invention providing an enhanced ability for geneticists to perform genetic selections based on the automated high-speed identification and genotyping of eggs results in more rapid genetic progress towards developing improved poultry lines.

20 Independent of which type of detection is utilized for genotypic classification, another contemplated design involves classifying each egg as "live" or "dead." Simple light and/or temperature mechanisms are also contemplated for this procedure as incorporated in existing systems by PAS Reform, Breuil, and Embrex. For this aspect
25 of the present invention, U.S. Patent No. 5,900,929 assigned to Embrex is incorporated herein by reference.

Coupled with the sorting devices described below, the present invention injecting the liquid formulation that provides a predetermined indication and the
30 detection thereof provides a versatile platform for all manner of molecular detection applications.

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Still another embodiment of the present invention incorporates a liquid sampling device for obtaining liquid samples from the avian eggs. This design uses a vacuum line in communication with a sampling needle, the tip of which is reciprocated to be surrounded by the liquid portion of a respective avian egg and then removed therefrom. Alternatively, the design may use an electro-osmotic gradient, similar to that utilized in the PE Biosystems 310 genetic analyzer, to draw fluid samples into a sampling capillary.

In order to utilize maximally the above-described detection mechanism(s), it may be necessary at certain times to amplify the signal by various techniques. One embodiment for the amplification of a detection signal could be the incorporation of an integrated thermal cycling unit, such as those produced by PE Biosystems, Hybaid MJ Research and others, for DNA amplification. This device would be important—if not essential—for the use of gene chip, micro-array, and macro-array based genotyping devices discussed above.

To obtain more detailed information about the samples being analyzed, it is also contemplated separating the sample molecules based on size, molecular weight, electric charge or other chemical/physical properties. One embodiment of this separations mechanism is an electrophoresis unit. For example, an integrated capillary electrophoresis unit such as or similar to the 310 Genetic Analyzer produced by PE Biosystems could be utilized to separate both nucleic acids and proteins. This embodiment of the present invention would, for instance, be useful for high-throughput genotyping of eggs from a primary breeder's pedigreed lines of poultry.

Ultimately, the data obtained from the above embodiments, either singularly or collectively, can be processed by software in a central processing unit or other device to evaluate the value and status of eggs as they come off the processing line. The evaluated eggs can be labeled, sorted, and transferred to hatching baskets or trays accordingly. Labeling can be performed by an ink jet mechanism, similar to that currently found in Hewlett Packard and Epson printers. Sorting and transfer of the eggs

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can be performed by automated suction cups and movable belts which transport trays of eggs through the instrument body and to the waiting egg carts. Mechanisms for this part of the instrument could use designs similar to systems manufactured and sold by Breuil, Kuhl Corporation, Pas Reform, and Embrex.

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The instrument platform being described here would also benefit from certain existing genetic/protein analysis capabilities. By directly incorporating the analysis capabilities into the platform these procedures could be performed at high-speed and at an industrial scale. Examples where these analysis capabilities would be directly applicable to the commercial poultry industry are described.

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Compared to the prior art systems, the present invention more efficiently manages poultry breeding operations. The present invention achieves this benefit by relying, in part, on the advent of In Ovo Fertilization technology along with linking together several important genetic technologies to create a truly versatile process and platform for the automation of poultry breeding technologies.

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Although the present invention has been described with reference to specific details of certain embodiments thereof, it is not intended that such details should be regarded as limitations upon the scope of the invention.

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METHOD OF FERTILIZING AN AVIAN EGG IN THE SHELL

BACKGROUND OF THE INVENTION

5 FIELD OF THE INVENTION

The present invention relates to the field of avian egg fertilization. In particular, the present invention relates to a method of fertilizing an egg in a shell. The invention also relates to a method of fertilizing an egg in a shell, whereby a live chick is hatched.

The invention also relates to an oviposited avian egg comprising a native embryo
10 having fewer than 40,000 cells, wherein the embryo can develop into a live chick.

BACKGROUND ART

It has long been a goal of avian geneticists to supplement traditional selection procedures by inserting desirable genes directly into the avian germline. Substantial
15 progress along these lines has been made in mammalian species where early embryos are accessible and where pronuclei can be visualized for the insertion of exogenous DNA. The production of transgenic mice, cattle, sheep, and goats has become a routine procedure in large commercial operations. In avian species, by contrast, the early embryo has not been easily accessed and the pronuclei are visually obscured, making
20 manipulation of the avian genome in early embryos *in ovo* an insurmountable goal.

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5 The difficulty involved in accessing an early stage avian embryo is best understood by understanding the anatomy of an avian egg and its progression through the female reproductive tract. At the time of ovulation, the avian oocyte comprises a blastodisc, or germinal disc, which contains the female pronucleus, and a yellow yolk mass. The germinal disc and yolk mass are surrounded by the oocyte cell membrane, called the oolemma. Surrounding the oolemma is the perivitelline layer (PL), also referred to as the inner perivitelline layer (IPL). The space between the oolemma and the IPL is termed the perivitelline space, which is traversed by granulosa cells. Once the oocyte is released from its ovarian follicle, it is referred to as an ovum. The ovum moves into the oviduct where it is engulfed by the infundibulum, where fertilization occurs if sperm are present.

15 As the ovum passes into the posterior infundibulum, another layer, the outer perivitelline layer (OPL), surrounds the ovum. This membrane acts to prevent polyspermy, which is a lethal condition that occurs when multiple sperm bind to and penetrate the ovum at the region of the blastodisc. The egg is then surrounded with additional layers of chalaza and thick and thin layers of albumen. When the ovum moves into the isthmus, two shell membranes are deposited, upon which small crystals of calcium carbonate are deposited, thus beginning the formation of the shell.

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The preceding events all occur within the first few hours following fertilization.

The ovum next moves into the uterus, where over the next 18-20 hours, the calcium shell is completed. The egg then moves into the vagina for several minutes, and then is extruded from the vagina, or oviposited (i.e., "laid"). At this point, if the egg has

5 been fertilized, the embryo contained therein will have 40,000 to 70,000 cells.

(Johnston ("In Vitro Sperm Binding, Penetration, and Fertilization of Recently

Oviposited Chicken Eggs," December 1988, Clemson University); Olsen, M.W., J.

Morph. 70: 413-533 (1942); Etches et al., in *Methods in Molecular Biology*, vol. 62

Recombinant Gene Expression Protocols, Ed. R. Tuan, Humana Press, Inc. Totowa, NJ,

10 pp. 433-450 (1997); Petite et al., in *Manipulation of the Avian Genome*, Ed. Etches et al., CRC Press, Boca Raton, FL, pp.81-101 (1993)).

The development of genetically modified birds largely requires access to early stage, pluripotent embryonic cells. As the earliest stages of embryo development occur

15 within the oviduct of the female, the introduction of heterologous DNA into the early embryo has only been possible by removing the embryo from the female, requiring that she be sacrificed. Furthermore, while culture of the early stage embryo to hatch has been accomplished, the method is extremely laborious, and results in a post-hatch survival rate of only about 10%. Sang et al., in *Manipulation of the Avian Genome*, Ed.

20 Etches et al., CRC Press, Boca Raton, FL, pp.121-133 (1993).

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In addition, a common technique employed by the poultry industry is to artificially inseminate females. While this technique can allow for an increase in fertility and reduces the number of males required in the breeding program, it is extremely labor intensive.

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The present invention provides a ground-breaking improvement in avian biology by making it possible to fertilize an egg in its shell, and to hatch a live bird from the shell. The methods of the invention, therefore, make it possible to access an embryo as early as a zygote. The methods also allow for the automation of *in ovo* fertilization.

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SUMMARY OF THE INVENTION

The present invention provides a method of fertilizing an avian egg in a shell, wherein the egg comprises a yolk enclosed by a membrane, comprising obtaining a sperm sample comprising avian sperm in a physiologically acceptable carrier, and delivering the sperm sample into the egg, so as to fertilize the egg.

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The present invention also provides a method of fertilizing an avian egg in a shell, wherein the egg comprises a yolk enclosed by a membrane, and hatching a live chick, comprising obtaining a sperm sample comprising avian sperm in a

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physiologically acceptable carrier, delivering the sperm sample into the egg, so as to fertilize the egg, incubating the egg, and hatching the live chick from the egg.

Also provided by the present invention is an oviposited avian egg comprising a native embryo having fewer than 40,000 cells, wherein the embryo can develop into a live chick. Relatedly, an oviposited avian egg comprising an embryo having fewer than 40,000 cells wherein the eggshell has an opening of less than 4 centimeters is also provided.

10 DETAILED DESCRIPTION OF THE INVENTION

As used herein, "a," "an" or "the" may mean one or more. For example, "an" egg may mean one egg or more than one egg. Moreover, "the" egg may mean one egg or more than one egg.

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The present invention provides a method of fertilizing an avian egg in a shell, wherein the egg comprises a yolk enclosed by a membrane, comprising obtaining a sperm sample comprising avian sperm in a physiologically acceptable carrier, and delivering the sperm sample into the egg, so as to fertilize the egg. The present

20 invention relates to the unexpected and surprising discovery that an unfertilized, oviposited avian egg can be fertilized in the shell. As used herein, reference to an avian egg in a shell refers to an oviposited egg, that is, an egg with a calcium carbonate shell

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that has been extruded from the vagina of the bird. Extrusion of the egg is referred to as "oviposition." Accordingly, all references herein to an "egg in a shell" or to an "oviposited egg" should be understood to be equivalent in meaning.

The process of fertilizing an oviposited egg in a shell is referred to herein as "in ovo fertilization" (IOF).

An avian egg comprises a hard, calcified shell at the time the egg is oviposited. Within the shell is a yolk that contains nutrients for supporting growth and development of an embryo. As used herein, an "embryo" is a developing organism resulting from the joining of a female pronucleus and a male pronucleus during the process of egg fertilization. While a fertilized (single cell) ovum may thus be called an embryo, the single cell embryo is also specifically referred to herein as a zygote.

Although in ovo fertilization can be performed on eggs as old as 2 weeks if the eggs are maintained at room temperature, ideally newly oviposited eggs are used for the best results. In a preferred embodiment, the sperm sample is delivered into the egg between 0 and 96 hours following oviposition. In a more preferred embodiment, the sperm sample is delivered into the egg between 0 and 72 hours following oviposition. In an even more preferred embodiment, the sperm sample is delivered into the egg between 0 and 48 hours following oviposition. In a highly preferred embodiment, the sperm sample is delivered into the egg between 0 and 24 hours following oviposition.

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Thus, it is preferred that the sperm sample be delivered into the egg as soon as possible following oviposition. However, the precise timing of fertilization can depend on how the oviposited egg is maintained, e.g., temperature, humidity, etc.

5 The sperm in the sperm sample may be obtained from a bird by methods known to a person skilled in the art, such as the abdominal massage method which is well-known to those of skill in the art. This method allows the collection of an ejaculate comprising sperm, seminal fluid, and transparent fluid. Transparent fluid is a lymphlike fluid that passes from the lymph channels to the surface of the phallus during
10 phallic tumescence. Avian sperm may also be obtained from commercial sources that are well known to those of skill in the art.

 In one embodiment, the sperm in the sperm sample can be collected from a single bird. In another embodiment, the sperm in the sperm sample is a mixture of
15 sperm obtained from more than one bird. When a mixture of sperm from more than one bird is used in the methods of the invention, the probability of successfully fertilizing the egg can increase, because if one of the birds from which the sperm has been collected is infertile, it is possible that the sperm collected from the other bird or birds will be capable of fertilizing the egg.

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 In a preferred embodiment, the sperm sample comprises sperm from birds

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which are members of the same species, and the sperm sample is used to fertilize eggs oviposited by hens which are members of the same species as the sperm donors. As one of ordinary skill is aware, individuals are classified as being from the same species if they are capable of mating to produce offspring. Moreover, the present invention
5 also contemplates the use of sperm from one species and an egg from another species, if the sperm is capable of fertilizing the egg.

While it is typically preferred that the sperm be used within 30 minutes of the time that it is collected, older sperm, and even sperm which have previously been
10 frozen, may be used in the methods of the invention, as long as the sperm retain their ability to fertilize an ovum. Where the sperm are to be used more than 30 minutes after collection, it is preferred that they be combined with a sperm extender, as is described below.

15 As mentioned above, the sperm sample also comprises a physiologically acceptable carrier. As used herein, a "physiologically acceptable carrier" is a fluid in which sperm remain motile and viable. Examples of a physiologically acceptable carrier include, but are not limited to, seminal fluid, transparent fluid, buffered saline solution, sperm extender, and combinations thereof. Preferably, the carrier will be a
20 sperm extender, also referred to in the art as a diluent. As mentioned above, the use of a sperm extender is especially preferred where the collected sperm will not be used for

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fertilization within 30 minutes after collection. M.R. Bakst, In *Manipulation of the Avian Genome*, R. J. Etches and AM. Verrinder Gibbons, eds., CRC Press, Boca Raton, FL, pp. 15-28 (1993). As used herein, a "sperm extender" is a physiologically acceptable carrier that is used to dilute a sperm sample to produce a sperm sample of greater volume in which the sperm are less concentrated. Preferably, the composition of the sperm extender will extend the shelf life of the sperm, as well as diluting the sperm so as to increase the number of eggs which may be fertilized by the quantity of sperm which has been collected. Examples of sperm extender compositions, suggested dilution rates, optimal storage times and conditions, and commercial sources of extender may be found in Bakst ("Preservation of Avian Cells: *In: Poultry breeding and Genetics*, R.D. Crawford (ed.) Elsevier, New York, pp 91-108 (1990)). Another example of a sperm extender is the semen diluent described by Johnston ("In Vitro Sperm Binding, Penetration, and Fertilization of Recently Oviposited Chicken Eggs," December 1988, Clemson University). Other diluents commonly used in the poultry industry are Lago Formulation Avian Semen Extender by Hygeia Biological Laboratories, Semaide Turkey Extender by Poultry Health Laboratories in Davis California, Beltsville Poultry Semen Extender by Tri Bio Laboratories, Inc. in State College, Pennsylvania. In a preferred embodiment, the sperm extender is Avidiluent. Avidiluent is produced by IMB, 10 rue Georges, Clemenceau, BP 81, 61302 l'Aigle, France.

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Thus, in one embodiment, the sperm sample may comprise sperm and seminal fluid, i.e., semen. Moreover, the sperm sample may comprise sperm and seminal fluid which is diluted with a physiologically acceptable carrier, including but not limited to buffered saline solution and a sperm extender.

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The sperm sample can also be prepared by methods which will be clear to one of ordinary skill in the art, such as washing semen from one or more birds with a solution such as buffered saline solution or sperm extender, centrifuging the resulting solution, removing the supernatant, and resuspending the washed sperm in a volume of a solution such as buffered saline or semen extender. One of ordinary skill in the art will readily understand how to achieve the desired concentration of sperm by resuspending the sperm in the appropriate volume of solution. For example, following centrifugation and removal of supernatant, the packed sperm may then be weighed, and the number of sperm then estimated by using known values for the weight of avian sperm. The sperm may then be resuspended in the volume required to obtain the desired sperm concentration. Alternatively, the centrifuged sperm may be resuspended following removal of the supernatant, and then recentrifuged, allowing the determination of the packed sperm volume. (Johnston, 1998). Subsequently, the concentration of the sperm may be calculated using the formula of Maeza and Buss. (Poultry Sci. 55:2059 (1976).)

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Typically, the concentration of sperm in chicken semen is from 300 million to 800 million per milliliter, in turkey semen from 800 million to 1.5 billion per milliliter, in Guinea fowl semen from 400 million to 800 million per milliliter, in Pekin duck semen from 20 million to 600 million per milliliter. The standard number of sperm
5 used for artificial insemination is 100 million in a total volume of 50 microliters. In the methods of the present invention, because sperm are placed directly adjacent to the female pronucleus, far fewer sperm are required to fertilize the egg. Thus, as few as one sperm can be used in the methods of the present invention. In fact, a large range of sperm concentrations can be used in the present invention. In one embodiment, chicken
10 semen is diluted with an equal volume of Avidiluent and approximately 0.01 milliliters of this sperm sample is injected into an egg. Thus, approximately 1 million sperm are deposited adjacent to the female pronucleus.

In the methods of the invention, fertilization of the avian egg in the shell is
15 accomplished by delivering the sperm sample into the egg. Delivery of the sperm sample may be accomplished by any method which will allow the sperm to be delivered inside the shell, including, but not limited to, dissolving an area of the shell with, e.g., an acid solution, using electroporation, and creating an opening by penetrating or cracking an area of the shell, for example using a tool such as a needle or
20 a scalpel.

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Preferably, the surface of the area of the shell to be penetrated in order to deliver the sperm sample is sanitized before the sperm is delivered inside, to prevent contamination of the egg. Any method which is compatible with the delivery method may be used to sanitize the shell, including, but not limited to, the disinfectant IOFEC-200, and 3% hydrogen peroxide. The surface of the egg at the intended penetration site may be wiped or sprayed with the disinfectant, or the egg may be immersed in a vessel containing the disinfectant of choice.

As is described above, an opening in the shell can be made with a tool such as a knife or a needle. Preferably, the tool will be sterile. For example, in a two-step procedure, an opening in the shell can first be made with a knife or other sharp instrument. In a second step, a needle attached to a syringe containing a sperm sample can be passed through the opening to deliver the sperm sample into the egg.

Introduction of the sperm sample into the opening in the shell may also be accomplished by other means, including, but not limited to, the use of a pipette, such as a micropipettor. Alternatively, in one step, a needle attached to a syringe containing the sperm sample can be used to penetrate and thus create the opening in the shell and deliver by injection the sperm sample into the egg. Thus, "opening" can include a hole created by a needle. Of course, one of ordinary skill in the art will be able to choose a needle whose gauge will be large enough to allow the sperm sample to be moved through the needle. In one embodiment, the needle will be of the smallest gauge that

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The opening can be made anywhere in the shell that effects viable fertilization, but is preferably made in an area of the shell that is near the germinal disc. While an egg may be manipulated so as to place the germinal disc at different regions of the egg, the germinal disc in a newly oviposited egg is located at the large end of the shell, which overlies the air cell adjacent to the yolk. Once an opening has been created in the shell, the sperm sample is preferably delivered by introducing the needle, pipette, etc., through the air cell and beyond a membrane lying below the air cell. In a further preferred embodiment, the sperm sample is delivered into the egg using a needle.

Preferably, the needle, pipette, etc., is advanced through the shell at an angle of approximately 15°, penetrating the membrane lining the shell. In a preferred method of practicing the methods of the invention, the needle, pipette, etc., is advanced through the air cell, until it meets a membrane which encloses the yolk and germinal disc. A

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person practicing the method of the invention will know that the tip of the needle, pipette, etc., has encountered the membrane when slight resistance to further advancement of the tip is felt. As the tip is gently advanced, the resistance from the membrane gives way and the tip is allowed to barely penetrate the membrane.

- 5 Preferably, the sperm sample is then delivered into the egg, adjacent to a region of the membrane and that is adjacent to the germinal disc. Typical volumes of the sperm sample are as small as 0.005 ml or as large as 0.10 ml. A preferred volume of injected sperm sample is about 0.01 ml.

- 10 Preferably, to prevent contamination of the egg and death of an embryo, the opening in the shell is sealed. A non-toxic adhesive can be applied directly to the opening in the shell to seal it. Alternatively, a piece of eggshell can be used as a patch to close the opening and may be attached to the shell with a non-toxic adhesive. In one embodiment, the non-toxic adhesive is Elmer's® glue. Moreover, any "tissue glue" can
15 also be used to seal the shell. A "tissue glue" is a sterile, non-toxic adhesive used during surgical, operative procedures to bind tissues together.

- The method of the present invention can be used to fertilize oviposited eggs from avian species selected from the group consisting of chicken, quail, duck, turkey,
20 pheasant, ostrich, emu, goose, peafowl, grouse, rhea, parrot, cockatiel, cockatoo, parakeets, and other commercially valuable birds.

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The present invention also provides a method of fertilizing an avian egg in a shell, wherein the egg comprises a yolk enclosed by a membrane, and hatching a live chick, comprising obtaining a sperm sample comprising avian sperm in a

5 physiologically acceptable carrier, delivering the sperm sample into the egg, so as to fertilize the egg, incubating the egg, and hatching the live chick from the egg.

After the sperm sample has been delivered into the egg according to the methods of the present invention as described above, the egg is incubated until the live

10 chick is hatched. One of ordinary skill will be aware of the amount of time and the preferred conditions for incubating a fertilized egg belonging to a particular species. The following are incubation periods for various species of birds: Chicken -21 days, Quail- 23 days, Cornish quail- 17 to 18 days, Pheasant-23days, Turkey-28 days, Duck- 28 to 33 days, Goose-28 to 30 days, Parakeet-18 days, Parrots-28 days, Dove-14 days,

15 Mynah-14 days, Finch-14 days, Button Quail-16 days, Valley Quail-21 to 22 days, Swan-30 to 37 days. Incubation of eggs fertilized by the methods of the present invention as compared to naturally fertilized eggs need differ only in that the length of incubation time must be lengthened to include the amount of time that the fertilized egg would have spent within the body of the female prior to oviposition. In a preferred

20 embodiment, the incubation period lasts from 21 to 23 days for chicken eggs. While one of ordinary skill in the art will readily be able to determine the optimal temperature

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for incubation of an egg from a particular species of bird, typically the incubation temperature is between 95° F and 100° F. A chicken egg will be incubated at about 99.5° F. In a more preferred embodiment, the temperature at which the chicken egg is incubated will be lowered as the egg nears the point of hatching. Thus, in a preferred
5 embodiment, a chicken egg is incubated at 99.5° F from day 1 of incubation to about day 18 of incubation, and at 98.5° F from day 19 of incubation to hatching.

As is well known in the art, the humidity level at which an egg is incubated can be important for bringing the egg to hatch. Thus, typically the egg is incubated at
10 between 75% and 90% humidity. Preferably, the egg is incubated at about 80% humidity. More preferably, the humidity level at which the egg is incubated will be raised as the egg nears the point of hatching. Thus, in a preferred embodiment, a chicken egg is incubated at 80% humidity from day 1 of incubation to about day 18 of incubation, and at 85% humidity from day 19 of incubation to hatching. In a specific
15 preferred embodiment, an egg is incubated at 99.5° F and 80% humidity from day 1 of incubation to about day 18 of incubation, and at 98.5° F and 85% humidity from day 19 of incubation to hatching. As is well known in the art, turning the eggs during incubation is useful for promoting growth of the embryo.

20 It is further preferred that the incubation of the eggs take place in a commercial incubator. Commercial hatchers and setters are produced by many companies including

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PAS Reform, Jamesway, Chickmaster, Buckeye, Cumberland, Petersime, Humidaire Incubator Co., etc. Preferably, the eggs are moved from a setter incubator to a hatcher incubator at about 3 days prior to hatch. The hatcher basket allows the egg to lie on its side where the chick can more easily pip out. This basket also allows the chick to walk about immediately after hatch, which is necessary for the chicks development and viability.

In another embodiment, the present invention provides an oviposited avian egg comprising a native embryo having fewer than 40,000 cells, wherein the embryo can develop into a live chick. "Native" means growing, living or produced in its place of origin. Thus, a native embryo is an embryo that develops and hatches the same shell in which the female pronucleus was formed. By the time an ovum which has been fertilized naturally has been oviposited, the developing embryo has between 40,000 and 70,000 cells. However, the egg of the present invention is fertilized after it has been oviposited in its shell; thus, an embryo developing in the egg of the present invention will at some time during incubation have fewer than 40,000 cells. In fact, at the moment of fertilization, the embryo in the egg of the present invention will have one cell and is a zygote. As the embryo grows within the egg, normal cell division will occur and the number of cells will increase. Thus, the fertilized, oviposited egg of the present invention will at some time during incubation comprise an embryo having, for example, 10,000, 20,000 or 30,000 cells, including less than and between these

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numbers of cells.

In another embodiment, the present invention provides an avian egg in a shell comprising an embryo having fewer than 40,000 cells, wherein the embryo can develop into a live chick, and wherein the shell has an opening of less than 4 centimeters. In another embodiment, the opening in the shell is less than 2 centimeters. In another embodiment, the opening in the shell is less than 1 centimeter. In one embodiment, the opening in the shell is only large enough to accommodate a 22-gauge needle. The embryo can be either native or non-native to the egg.

10

An egg of the present invention may, for example, be derived from avian species selected from the group consisting of chicken, quail, duck, turkey, pheasant, ostrich, emu, goose, peafowl, grouse, rhea, parrot, cockatiel, cockatoo, parakeets, swan, dove, and other commercially valuable birds. In a commercially preferred embodiment, the egg is derived from avian species used in the methods of the present invention and is selected from the group consisting of chicken, turkey, goose, duck, quail, and pheasant. In a more preferred commercial embodiment, the egg is derived from a chicken. The method can also be effectively utilized on avian species in zoos, e.g., to help preserve endangered species, including but not limited to those found in

20 McGrew's zoo.

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The methods of the present invention can also be used for in ovo fertilization of reptilian eggs. Reptilian eggs, similar to avian eggs, comprise a yolk and female pronucleus and are protected by a shell when they are laid. An unfertilized, oviposited reptilian egg can be fertilized in the shell according to the methods of the present invention. In particular, a sperm sample, comprising sperm from one or more reptiles of the same species, is delivered into the unfertilized, oviposited egg through an opening created in the shell and onto the yolk adjacent to the female pronucleus where fertilization occurs.

10 The present invention also provides a method for producing an avian embryo containing heterologous nucleic acid comprising, fertilizing an avian egg by the method of the present invention disclosed herein, and introducing heterologous nucleic acid into the avian egg. As used herein, nucleic acids include, but are not limited to, DNA and RNA. The nucleic acids may be single, double, or multiple-stranded.

15

After an avian egg has been fertilized by IOF, the developing embryo can be accessed at any stage of development to manipulate the genetic makeup of some or all of its cells. Of greatest interest for creating transgenic birds is the early embryo. IOF allows for germ line transmission of the heterologous nucleic acid as it allows the earliest possible introduction of heterologous nucleic acid into the early avian genome. This genetic manipulation of the developing embryo can, for example, produce

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transgenic birds comprising genetic material which can be used to modulate endogenous DNA and its expression and to create cells which can manufacture commercially valuable proteins for commercial use.

- 5 Transfer of nucleic acid into the avian genome can be performed by a person skilled in the art according to several known methods. (Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1988; Lennette et al., *Manual of Clinical Microbiology*, 14th Ed., Amer. Soc. for Microbiology, Washington, D.C., 1985; Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989; *Antisense RNA and DNA*, D. A. Melton, Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1988)) Freshney, *Culture of Animal Cells, A Manual of Basic Technique*, 2nd Ed. Alan R. Liss, Inc., New York (1987); Centers for Disease Control Laboratory Manual, U.S. Department of Health, Education, and
- 10 Welfare Pub. No. 79-8375, p. 75, Centers for Disease Control, Atlanta, GA; *Fundamental Virology, 2nd Ed.* Bernard N. Fields and David M. Knipe, Chief Eds., Raven Press: New York, 1990). Examples of methods of transferring genetic material into the avian genome include, but are not limited to, avian leukosis virus (ALV) transduction mediated transgenesis, transposon mediated transgenesis, blastodermal cell
- 15 mediated transgenesis, primordial germ cell mediated transgenesis and nuclear transfer. The heterologous nucleic acid may be introduced into the embryo at the same time that
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the sperm sample is introduced into the egg, or at a later stage of embryonic development.

A standard method now in use for producing transgenic birds is to produce replication deficient ALV-derived transducing particles comprising a DNA insert. Because the particles are not capable of replicating in the bird, there is no risk of causing viremia and illness in the bird or humans. A window can be generated just above the embryo and transducing particles introduced into the subgerminal cavity of the embryo. The window is then sealed, and the eggs are placed into incubators for development.

Another method used to transfer heterologous nucleic acid into the avian genome is transposon-mediated transgenesis. Transposons are genetic elements that are able to translocate or move about within the genome of their host species. There are no known transposons that occur in avian species. However, it has been shown that certain transposons, reconstructed from other sources, are able to function within the avian genome. These transposons can be engineered to serve as useful nucleic acid vector systems for inserting genes into the avian genome.

20 Further, another method for introducing heterologous DNA into an avian embryo is blastodermal cell transgenesis. Blastodermal or other early cells can be

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taken from stage X embryos (the embryonic stage at which a fertilized egg is laid) can be injected back into recipient embryos where they colonize and grow, giving rise to chimeric chickens. A "chimeric" chicken is composed of cells from two different genetic lineages. Blastodermal cells injected into irradiated host embryos soon after they are isolated have the ability to contribute to all tissues of the resulting bird including the germline (cells that give rise to sperm and eggs and thus to all following generations). Blastodermal cells have been cultured and transfected with DNA by various methods known to persons skilled in the art in an attempt to generate transgenic poultry.

10

Thus, blastodermal cells or embryonic stem (ES) cells can be injected into an in ovo fertilized embryo to create chimeric poultry. These chimeric poultry can then be crossed and, assuming they have germ line transmission of the blastodermal or ES cells, one can create a clone of the original injected cell.

15

Moreover, primordial germ cell mediated transgenesis may be used to create transgenic birds. As the developing embryo grows, certain cells are committed to the germline to give rise to the sperm and eggs. These primordial germ cells (PGCs) migrate to the genital ridge of the developing gonad where they lie dormant until the bird reaches sexual maturity. One approach to avian transgenesis is to isolate these PGCs, genetically manipulate them and place them back into a developing embryo for

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continued development. This technical approach is comparable to the blastodermal cell approach in both the extent to which it is developed and its potential applications.

Primordial germ cells have been shown to contribute to the germline when injected into recipient embryos just as blastodermal cells have.

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Nuclear transfer is another technique by which a person skilled in the art can produce a transgenic bird. Nuclear transfer involves the transfer of a nucleus from a donor cell (nucleoplast) into an enucleated cytoplasm (cytoplast) to form a reconstituted cell with the genetic makeup of the donor nucleus. Nuclear transfer has many scientific applications; however, the most widely publicized application is for the cloning of animals. Initially nuclear transfer was performed to derive clones from a single embryo to produce several identical cloned animals. Typically nuclei from an embryo at the 8 to 64-cell stage are introduced into enucleated oocytes to produce new single-cell embryos capable of development to form cloned animals. Clearly, the fertilization methods and fertilized avian eggs having less than 40,000 cells would make such work much easier, as the 8-64 cell embryo could easily be accessed without the necessity of sacrificing the female bird giving rise to the ovum. Nuclei from somatic cells such as fibroblasts and mammary epithelial cells can be combined with an enucleated oocyte where they are reprogrammed by cytoplasmic factors to become a new viable embryo capable of giving rise to a healthy animal.

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The in ovo fertilization methods described herein can also be utilized in conjunction with other in ovo procedures. For example, the embryo can be vaccinated after fertilization. Alternatively, such vaccination could occur simultaneous with in ovo fertilization providing however that the vaccine did not prevent development of the embryo.

Additionally, in ovo fertilization can be automated such that multiple eggs are simultaneously fertilized by, for example, injection techniques.

It will be clear to those of ordinary skill in the art that the methods of the present invention may easily be applied to a large-scale industrial operation, using automation to fertilize newly laid eggs. Accordingly, an apparatus which has previously been used, for example, to immunize newly fertilized eggs can be adapted to instead introduce a sperm sample.

The following Example is set forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how the methods claimed herein performed, and is intended to be purely exemplary of the invention and is not intended to limit the scope of what the inventors regard as their invention. Efforts have been made to ensure accuracy with respect to numbers (e.g., amounts, temperature, etc.) but

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some errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, temperature is in °F and pressure is at or near atmospheric.

EXAMPLE

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1. Forty-three freshly laid unfertilized chicken eggs were disinfected by wiping the shells with 3% hydrogen peroxide and placed in racks.

2. Sperm was obtained from 4 barred rock roosters on the same morning and collected in Vacutainer® vials less than 1 hour before the fertilization procedure was performed.

3. Sperm was pooled from the 4 roosters and mixed with 1 ml of Avidiluent®.

4. Sperm mixed with Avidiluent® was drawn into a 1 ml syringe through a 1", 22 gauge needle to form a sperm sample.

5. The needle created an opening in the large, blunt end of the eggshell and passed through the opening at a 15° angle to the surface of the shell.

6. The needle was passed through the air cell until the tip just penetrated the membrane enclosing the yolk and germinal disc.

7. One drop, 0.05 ml, of the sperm sample was injected onto the surface of the yolk adjacent to the membrane.

8. The needle attached to the syringe was withdrawn from the egg.

9. The opening created in the shell by the needle was patched with a small piece of

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shell, and the patch was secured to the shell with an adhesive such as Elmer's glue®.

10. The eggs were placed in commercial grade settlers maintained at 99.5°F and 80% humidity from day 1 to about day 18 of incubation. The eggs were turned according to methods known in the art and used in commercial settlers.

11. On day 19, the eggs were transferred to commercial hatchers and maintained at 98.5°F and 80% humidity until hatching.

Ten days after the fertilization method of the present invention was performed on 43 eggs, routine candling of the eggs was performed to determine which eggs had been successfully fertilized. Thirty-five eggs of the 43 eggs had been fertilized. Of the 35 fertilized eggs, 32 were successfully brought to hatching, and all but one of the chicks were healthy. Thus, 72% of the 43 oviposited eggs treated by the fertilization method of the present invention produced a healthy live chick.

Throughout this application, various publications are referenced. The disclosures of these publications, and the references cited therein, in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

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It will be apparent to those skilled in the art that various modifications and variations can be made in the present invention without departing from the scope or spirit of the invention. Other embodiments of the invention will be apparent to those skilled in the art from consideration of the specification and practice of the invention disclosed herein. It is intended that the specification and example be considered as exemplary only, with a true scope and spirit of the invention being indicated by the claims.

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What is claimed is:

1. A method of fertilizing an avian egg in a shell, wherein the egg comprises a yolk enclosed by a membrane, comprising:

*general
known*

- a) obtaining a sperm sample comprising avian sperm in a physiologically acceptable carrier; and
- b) delivering the sperm sample into the egg, so as to fertilize the egg.

2. The method of claim 1, wherein the egg and the sperm are derived from members of a same species.

3. The method of claim 1, wherein the carrier is diluted seminal fluid.

4. The method of claim 3, wherein the diluted seminal fluid comprises Avidiluent.

5. The method of claim 1, wherein the sperm sample is delivered adjacent to a membrane enclosing the yolk.

6. The method of claim 5, wherein the sperm sample is delivered adjacent to a region of the membrane that is adjacent to the germinal disc.

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7. The method of claim 2, wherein the avian species is selected from the group consisting of chicken, quail, duck, turkey, pheasant, ostrich, goose, and rhea.
8. The method of claim 7, wherein the avian species is chicken.
9. The method of claim 1, wherein the sperm sample comprises a mixture of sperm obtained from more than one bird.
10. The method of claim 1, wherein the delivery of the sperm sample is achieved by creating an opening in the shell by penetrating the shell and introducing the sperm sample into the egg.
11. The method of claim 10, wherein the opening is sealed.
12. The method of claim 11, wherein the opening is sealed with an adhesive.
13. A method of fertilizing an avian egg in a shell, wherein the egg comprises a yolk enclosed by a membrane, and hatching a live chick, comprising:
 - a) obtaining a sperm sample comprising avian sperm in a physiologically acceptable carrier;
 - b) delivering the sperm sample into the egg, so as to fertilize the egg;

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- c) incubating the egg of step b); and
- d) hatching the live chick from the egg.

14. The method of claim 13, wherein the egg and the sperm are derived from members of a same species.

15. The method of claim 13, wherein the carrier is diluted seminal fluid.

16. The method of claim 15, wherein the diluted seminal fluid comprises Avidiluent.

17. The method of claim 13, wherein the sperm sample is delivered adjacent to a membrane enclosing the yolk.

18. The method of claim 17, wherein the sperm sample is delivered adjacent to a region of the membrane that is adjacent to a germinal disc.

19. The method of claim 13, wherein the avian species is selected from the group consisting of chicken, quail, duck, turkey, pheasant, ostrich, goose, and rhea.

20. The method of claim 19, wherein the avian species is chicken.

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21. The method of claim 13, wherein the sperm sample comprises a mixture of sperm obtained from more than one bird.
22. The method of claim 13, wherein the delivery of the sperm sample is achieved by creating an opening in the shell by penetrating the shell and introducing the sperm sample into the egg.
23. The method of claim 22, wherein the opening is sealed.
24. The method of claim 23, wherein the opening is sealed with an adhesive.
25. The method of claim 13, wherein the egg is a chicken egg, and incubation of the egg lasts from 21 to 23 days.
26. The method of claim 25, wherein incubation is performed at a temperature of about 99.5°F from day 1 to about day 18 of incubation and a temperature of about 98.5°F from about day 19 of incubation to hatching.
27. The method of claim 25, wherein incubation is performed at 80% humidity from day 1 to hatching.

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37. The egg of claim 34, wherein the opening is sealed.
38. The egg of claim 34, wherein the opening is less than 1 centimeter.
39. The egg of claim 34, wherein the opening is less than 5 millimeters.
40. An egg produced by the method of claim 1, wherein the egg comprises a zygote.
41. A method for producing an avian embryo containing heterologous nucleic acid comprising:
- a) fertilizing an avian egg by the method of claim 1; and
 - b) introducing heterologous nucleic acid into the avian egg.
42. The method of claim 41, wherein the heterologous nucleic acid encodes a pharmaceutical protein, an antigen, a hormone or an antibody.
43. The method of claim 41, wherein the heterologous nucleic acid comprises Avian Leukemia Virus-derived transducing particles.

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Abstract of the Invention

The present invention relates to the field of avian egg fertilization. In particular, the present invention provides a method of fertilizing an egg in a shell. The invention
5 also provides a method of fertilizing an egg in a shell, whereby a live chick is hatched. The invention also provides an oviposited avian egg comprising a native embryo having fewer than 40,000 cells, wherein the embryo can develop into a live chick.

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LOS ESTADOS UNIDOS DE AMERICA

**A TODOS AQUELLOS A QUIENES LA PRESENTE
VINIERE:**

DEPARTAMENTO DE COMERCIO DE LOS ESTADOS UNIDOS
Oficina de Patentes y Marcas de los Estados Unidos

Marzo 27, 2001

Por la presente se certifica que lo que obra anexo a ésta es copia fiel de los registros en la Oficina de Patentes y Marcas de los Estados Unidos de aquellos escritos de la solicitud de patente más adelante identificada que reunió los requisitos para que se le concediera una fecha de presentación sobre la base de 35 USC 111.

Número de Solicitud: 60/182,969

Fecha de Presentación: Febrero 16, 2000

**Por autoridad del
Comisionado de Patentes y Marcas**

**W. MONTGOMERY
Funcionario certificante**

(Hay un sello-oblea)

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REIVINDICACIONES

1. Un método para fertilizar un huevo de ave en su cáscara, donde el huevo comprende un vitelo recubierto por una membrana y comprende:
 - a) obtener una muestra de esperma que comrende un esperma de ave en un
5 portador fisiológicamente aceptable y
 - b) administra la muestra de esperma dentro del óvulo, para así fertilizar el huevo.
2. El método de la reivindicación 1, donde el huevo y el esperma se derivan de miembros de la misma especie..
- 10 3. El método de la reivindicación 1, donde el portador es un líquido seminal diluido.
4. El método de la reivindicación 1, en donde el líquido seminal diluido incluye Avidiluent.
- 5 El método de la reivindicación 1, en donde la muestra de esperma se
15 administra en forma adyacente a una membrana que encierra el vitelo.
6. El método de la reivindicación 5, en el que la muestra de esperma se administra en forma adyacente a una membrana adyacente a un disco germinal.
7. El método de la reivindicación 2, en donde la especie de ave es
20 seleccionada del grupo que consiste en gallinas, codornices, patos, pavos, faisanes, avestruces, gansos y fíandúes.
8. El método de la reivindicación 7, en donde la especie avícola es la gallina.
9. El método de la reivindicación 1, donde la muestra de esperma
25 comprende una mezcla de esperma obtenido de más de un ave.

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10. El método de la reivindicación 1, en donde la introducción de la muestra de esperma se efectúa practicando un orificio en la cáscara penetrando la misma e introduciendo la muestra de esperma en el huevo.
11. El método de la reivindicación 10, en el que el orificio es sellado.
- 5 12. El método de la reivindicación 11, en el que el orificio es sellado con un adhesivo.
13. Un método de fertilización un huevo puesto de ave dentro de su cáscara, en el que el huevo comprende un vitelo recubierto por una membrana, e incubar un pollo que comprende:
 - 10 a) la obtención de una muestra de esperma que incluye esperma avícola en un vehículo fisiológicamente aceptable; y
 - b) la administración de la muestra de esperma al interior del huevo, a fin de fertilizar el mismo.
 - c) incubar el huevo de la etapa b) y
 - 15 d) sacar del cascarón el pollo
- 14 El método de la reivindicación 13, donde el huevo y el esperma se derivan de miembros de la misma especie.
15. El método de la reivindicación 13, donde el vehículo es líquido seminal diluido.
- 20 16. El método de la reivindicación 15, donde el fluido seminal diluido comprende Avidiluent.
17. El método de la reivindicación 13, en el que la muestra de esperma es administrada en forma adyacente a una membrana que encierra el vitelo.
18. El método de la reivindicación 17, en el que la muestra de esperma es
25 administrada en forma adyacente a un sector de la membrana que es adyacente al disco germinal.

19. El método de la reivindicación 13, en el que la especie de ave es seleccionada del grupo que consiste en gallinas, codornices, patos, pavos, faisanes, avestruces, gansos y ñandúes.
20. El método de la reivindicación 19, en el que la especie avícola es la
5 gallina.
21. El método de la reivindicación 13, en el que la muestra de esperma comprende una mezcla de esperma obtenido de más de un ave.
22. El método de la reivindicación 13, en el que la introducción de la muestra de esperma se efectúa practicando un orificio en la cáscara penetrando
10 la misma e introduciendo la muestra de esperma en el huevo.
23. El método de la reivindicación 22, en el que el orificio es sellado.
24. El método de la reivindicación 23, en el que el orificio es sellado con un adhesivo.
25. El método de la reivindicación 13, donde el huevo es un huevo de
15 gallina, y la incubación del huevo dura de 21 a 23 días.
26. El método de la reivindicación 25, donde la incubación se lleva a cabo a una temperatura de 99.5°F desde el primer día de incubación hasta el día 18 y una temperatura alrededor de 98.5°F desde el día 19 de incubación hasta el nacimiento.
- 20 27. El método de la reivindicación 25, donde la incubación se lleva a cabo a una humedad de 80% desde el primer día hasta el nacimiento.
28. El método de la reivindicación 13, que adicionalmente comprende vacunación del embrión durante el paso (C).
29. Un huevo de ave puesto que comprende un embrión nativo con menos
25 de 40.000 células, en el que el embrión puede convertirse en un polluelo.

30. El huevo de la reivindicación 29, en el que el embrión presenta menos de 30.000 células.
31. El huevo de la reivindicación 29, donde el embrión es un cigoto.
32. El huevo de la reivindicación 29, que además comprende un ácido nucleico heterólogo.
33. El huevo de la reivindicación 29, el cual es un huevo de gallina.
34. Un huevo de ave puesto que comprende un embrión que tiene menos de 40000 células, donde la cáscara del huevo tiene un orificio de menos de 4 centímetros.
35. El huevo de la reivindicación 34, donde el embrión tiene menos de 30,000 células.
36. El huevo de la reivindicación 34, donde el embrión es un cigoto.
37. El huevo de la reivindicación 34, donde el orificio está sellado.
38. El huevo de la reivindicación 34, donde el orificio es menos de 1 centímetro.
39. El huevo de la reivindicación 34, donde el orificio es menos de 5 milímetros.
40. Un huevo producido por el método de la reivindicación 1, donde el huevo comprende un cigoto.
41. Un método para producir un embrión de ave que contenga ácido nucleico heterólogo que comprende:
- a) fertilizar un huevo de ave por el método de la reivindicación 1; y
 - b) introducir el ácido nucleico heterólogo dentro de un huevo de ave.
42. El método de la reivindicación 41, en el que el ácido nucleico heterólogo codifica una proteína farmacéutica, un antígeno, una hormona o un anticuerpo.

43. El método de la reivindicación 41, en el que el ácido nucleico heterólogo incluye el Avian Leukemia Virus derivado de partículas transducing.

2do. EXAMEN DE FORMA PATENTE DE INVENCION

Folio 179

Radicación no 01-11984

Concepto Técnico no 230 Clasificación Internacional de Patentes A 61 D19/02

Practicado el examen de acuerdo a lo establecido en el Art. 38 de la Decisión 486 se determinó que esta solicitud de Patente de Invención:

- | | | |
|--|-----------------------------|---|
| SI ☒ | NO ☐ | Contiene un resumen de la invención (Art.26-e). |
| SI ☒ | NO ☐ | Contiene el título o nombre de la invención (Art.27-d). |
| SI ☒ | NO ☐ | Contiene la descripción de la invención (Art.26-b). |
| SI ☐ | NO ☐ | NO ES APLICABLE ☒ Contiene los dibujos necesarios para comprender la invención (Art.26-d) |
| SI ☒ | NO ☐ | Contiene una o más reivindicaciones. (Art.26-c). |
| SI ☐ | NO ☐ | NO ES APLICABLE ☒ Han sido desarrollados los productos y/o procedimientos a partir de recursos genéticos o de sus productos derivados de los que cualquiera de los Países Miembros es país de origen. (Art. 26- h). |
| SI ☐ | NO ☐ | NO ES APLICABLE ☒ Los productos y/o procedimientos han sido obtenidos o desarrollados a partir de los conocimientos tradicionales de las comunidades indígenas, afroamericanas, o locales de los países miembros, de acuerdo a lo establecido en la Decisión 391 y sus modificaciones y reglamentaciones vigentes.(Art.26-f). |
| SI ☒ | NO ☐ | La invención se refiere a un producto relativo a un material biológico. (Art.26-J). |
| SI ☒ | NO ☐ | La prioridad coincide con la materia reivindicada. |
| SI ☒ | NO ☐ | CUMPLE LOS REQUISITOS TÉCNICOS |

OBSERVACIONES Y OTROS: SI ☐ NO ☒ ver hoja(s) anexa(s)

EXAMINADOR TÉCNICO
202049

Bogotá D. C., ENERO 24 DE 2003



2020
Bogotá DC

Doctor
MARGARITA CASTELLANOS ABONDANO
Apoderado(a)

REFERENCIA:	Radicación No.	01- 11964
	Trámite	002
	Evento	001
	Actuación	416
	Oficio No.	352
	Folios	001

Teniendo en cuenta que la solicitud de privilegio de patente que se tramita bajo el expediente indicado en la referencia, reúne los requisitos de forma establecidos en los artículos 26 y 27 de la Decisión 486 de la Comisión de la Comunidad Andina, y en las demás disposiciones vigentes sobre la materia, se envía el extracto de la solicitud a la Oficina de Comunicaciones para efecto de su publicación en la Gaceta de Propiedad Industrial, conforme lo establece el artículo 40 de la misma Decisión.

Cúmplase
Dado en Bogotá DC, a los

14 FEB. 2003


ALIX CARMENZA CÉSPEDES DE VERGEL
Jefe de la División de Nuevas Creaciones

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