In ovo sexing techniques: current and future developments

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Context of the issue in the poultry sector

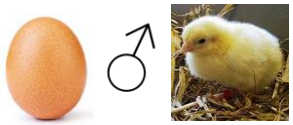
- Productions of table eggs and Foie gras
- Methods used to kill one-day-old chicks
- Alternatives

When phenotypic and physiological characteristics of one sex does not meet productivity criteria and/or product quality

Gallus gallus species

Laying hens (Egg-laying strains)
Table eggs

Selection of layers on egg quality and egg-laying performances



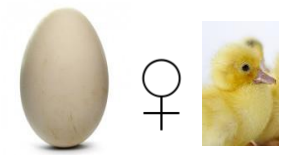
Culling of one-day old male chicks (no eggs and low meat yield and quality ☹)
=50 millions chicks per year in France

When phenotypic and physiological characteristics of one sex does not meet productivity criteria and/or product quality

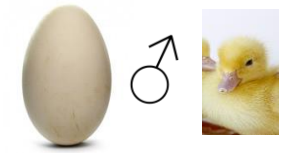
Anas platyrhynchos species

Barbarie + mulard ducks
Foie gras

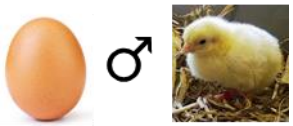
Selection of breeders on foie gras quality and yield



Culling of one-day old female chicks
(low foie gras quality and yield ☹)
≈15 millions ducklings per year in France



Sexing one-day old birds



Sorting after hatching

Egg-laying strains, *Gallus gallus*
-anatomical differences of the cloaca
-difference in the size of remiges or feather colour (brown strains)

♀ ♂

♀ ♂

"Fat liver" strains, *Anas platyrhynchos*
-autosexing strain (eye colour: red for females, black for males)

♀ ♂

≈50% female ducklings are reared to be sold as farm cans, for roasting (export)

Methods used for culling chicks and fate of dead birds

AVMA
American Veterinary Medical Association

Based on AVMA recommendations

Grinding/crushing using a specific dedicated equipment
Gas inhalation (CO_2 at 75% for 5 minutes)

After CO_2 treatment: Wildlife parks, animal rescue associations
After maceration : pet food processing plant

A highly publicized awareness, followed by ministerial announcements to ban the culling of chicks in various European countries

January. 2020

May 2021 (start in 2022)

Feb. 2022

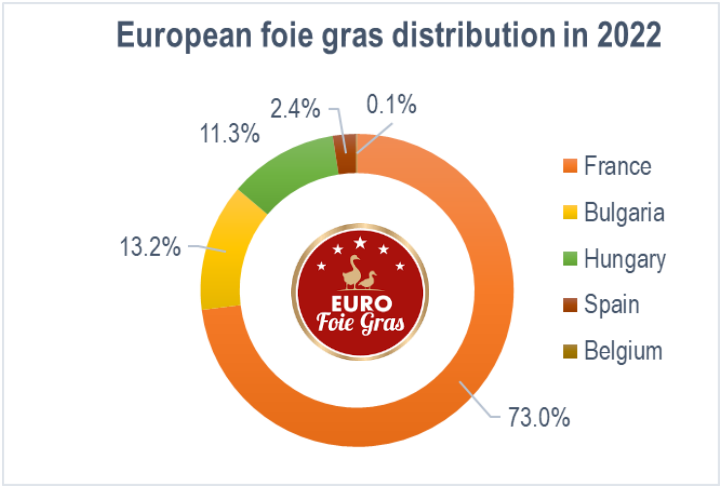
Légifrance BÊTA
Le service public de la diffusion du droit

Décret n° 2022-137 du 5 février 2022 relatif à l'interdiction de mise à mort des poussins des lignées de l'espèce Gallus gallus destinées à la production d'œufs de consommation et à la protection des animaux dans le cadre de leur mise à mort en dehors des établissements d'abattage

Luxembourg, Finland, Cyprus, Ireland, Spain, Belgium,...

What about the foie gras industry?

Stop culling female ducklings by 2025?



PPILOW Three possible alternatives

1 Rearing brothers of laying hens (actual layer strains)

No market today but initiatives in progress
In Germany: export of ♂ chicks to Poland (sausage and reconstituted meat)

2 Dual-purpose crossbreeds

In development for organic productions
High cost, quality of by-products to be optimized
-Hardly applicable to conventional channels at present

3 *In ovo sexing*

Deployed in all five hatcheries in France
No major change in the organization of the egg industry ; no need to develop new markets in the broiler poultry sector; concentration of efforts at hatcheries

In ovo sexing methods

- Sexual dimorphism *in ovo*
- Principle and challenges of ovosexing techniques
- Overview of ovosexing methods: the French case

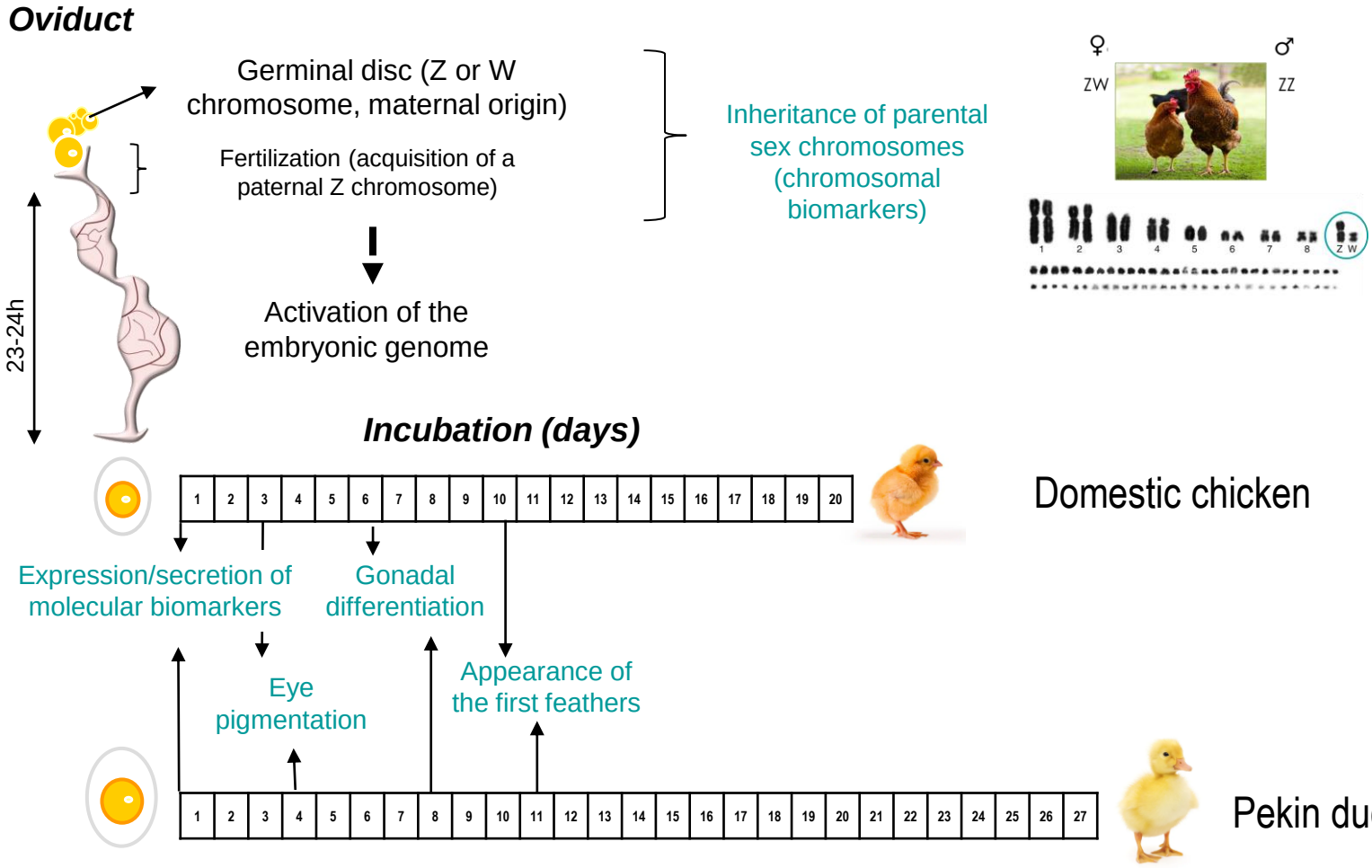
PPILOW Principles

The development of ovosexing techniques is based on the detection of differences between eggs containing an ♂ embryo or an ♀ embryo
= sexual dimorphism

1. genetic biomarkers (sex chromosomes)
2. anatomical features (development of gonads)
3. phenotypic traits (feather colour, growth kinetics ?, others ?)
4. molecular characteristics (lipids, proteins, genes, hormones, metabolites, volatile compounds)

Some of these features result from the activation of the genome and embryonic metabolism, and are visible/detectable lately in the incubation process

PPILOW Sexual dimorphism *in ovo* (*Gallus gallus* and *Anas platyrhynchos*)



For Foie Gras production: Barbarie and mullard ducks (31 and 35 days of incubation, respectively)

PPILOW Development of *in ovo* sexing methods in practice

Requirements : to be able to identify the sex of the embryo (using at least one of the sex dimorphic features) at the targeted time of incubation

Initial procedure

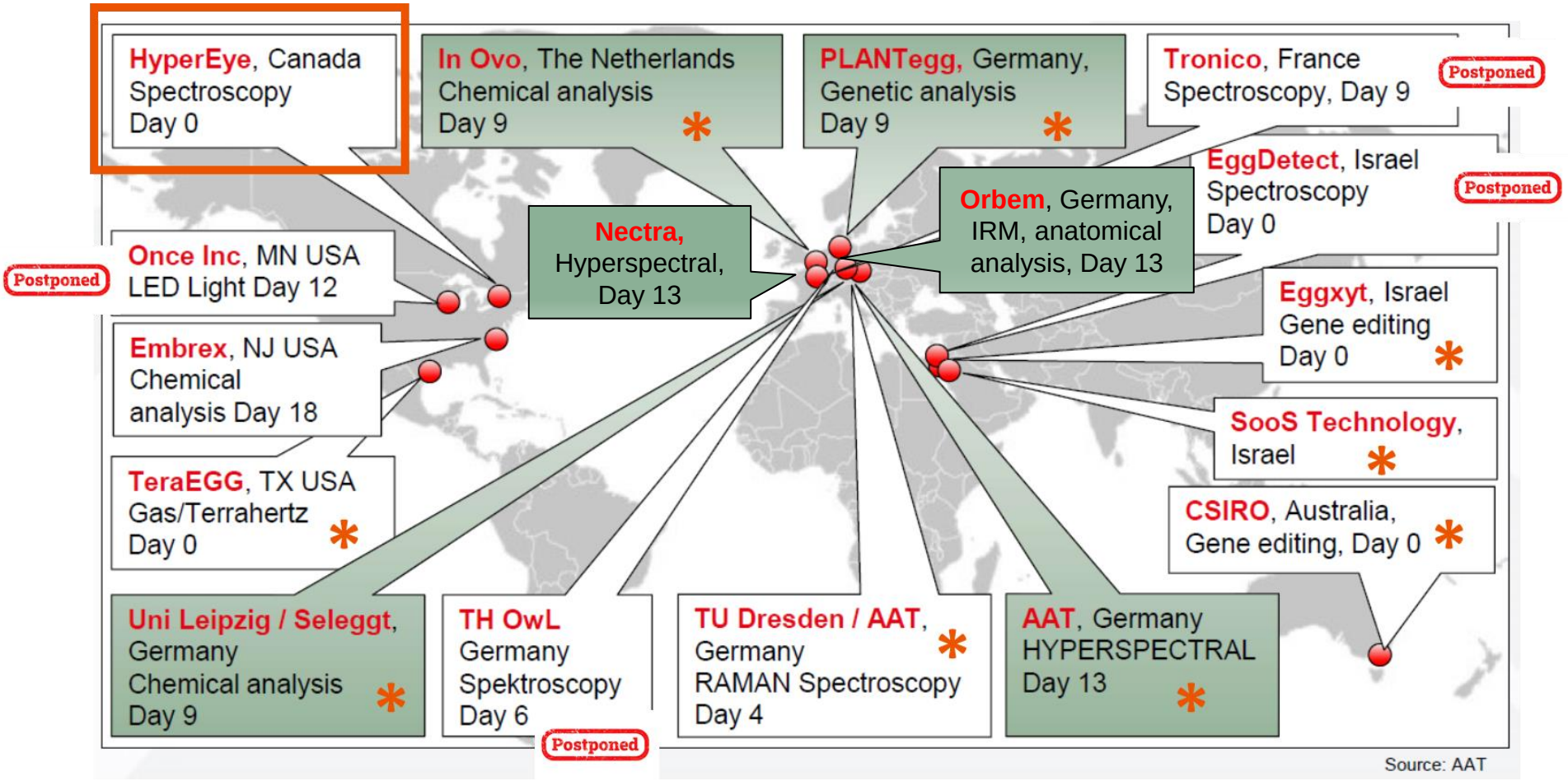
Step 1: Search for sex indicators / biomarkers (embryo / egg structures)

Step 2: Validate the markers on a large number of eggs from genetically different breeds with varying characteristics (weight/shape/shell colour, storage and incubation conditions)

Challenges

- Detection as early as possible (ideally ≤ 6 days of incubation) because of the controversy related to when the embryo acquires the ability to feel pain during its development
- Non-invasive (no impact on the development and viability of the embryo and animals after hatching)
- Fast, accurate, and effective
- Applicable in the field, on a variety of strains
- With acceptable cost for both professionals and consumers

PPILOW Overview of the methods that have been developed worldwide



Six techniques are marketed

PPILOW Sexual dimorphism associated with sex chromosomes

PLANTegg

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20

Chromosomes

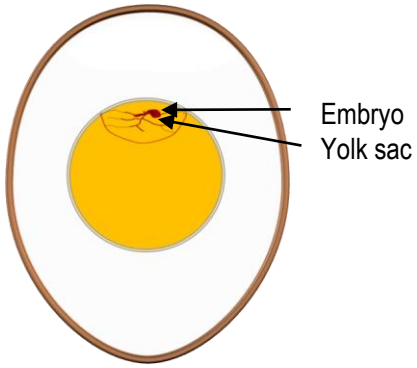
?

→ Detection of genes located on Z and/or W sex chromosomes

Characteristics: collection of cells contained in the allantoic fluid (invasive) / all breeds

→ The allantoic fluid does not develop until the 6th day of incubation

→ Other structures contain DNA before day 6 (embryo, yolk sac) but collection process is predicted to be highly invasive



3 days

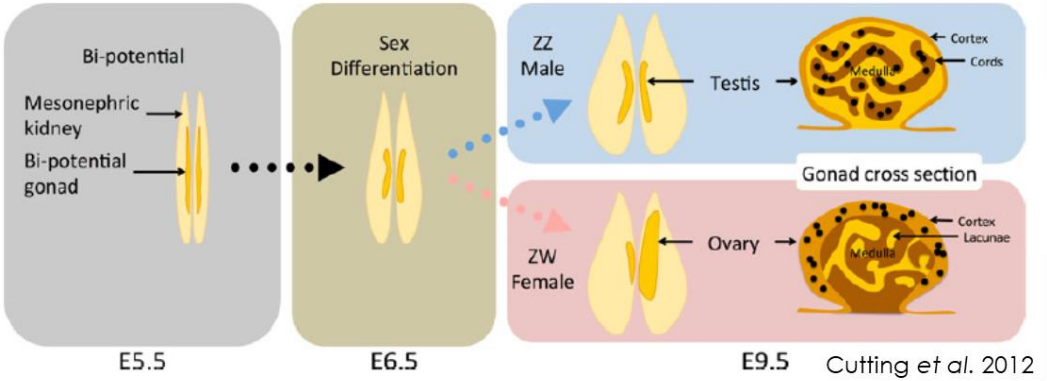
PPILOW Sexual dimorphism associated with anatomical differences (gonad development)

ORBEM

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20

Anatomy (gonads) ? → Magnetic resonance imaging (MRI)

Characteristics: non invasive, all breeds



→ Morphological difference detectable after dissection, as early as 9.5 days of incubation

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PPILOW Sexual dimorphism associated with phenotypic differences (feather colour)

♀ ♂

HENDRIX GENETICS

AAT EW GROUP COMPANY

NEC TRR

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20

Phenotype
(feather colour)

?

Hyperspectral methods
Characteristics: non-invasive / Brown breeds

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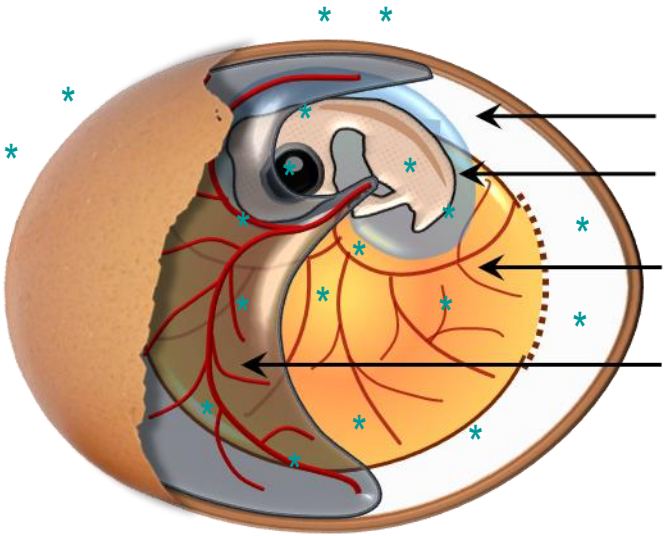
PPILOW Sexual dimorphism associated molecular differences (genes, proteins, metabolites, lipids, hormones, volatile compounds)

All breeds

→ Difference in yolk and egg white assimilation between ♂ and ♀ (linked to different ♂ and ♀ metabolisms)

→ Excretion/secretion/diffusion of ♂ and ♀ molecules (*) in yolk/white, through the shell, and into extra-embryonic structures

Distinct molecular profiles



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Egg white
Amniotic sac (amniotic liquid) — Day 3

Yolk sac — Day 1

Allantoïc sac
-allantoic fluid
-chorioallantoic membrane — Day 5

PPILOW Sexual dimorphism associated with molecular differences
(genes, metabolites, lipids, hormones, volatile compounds)

SELEGGT
responsible solutions

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20

Quantification of estrone sulfate (female-specific) in allantoic fluid)
Characteristics : semi-invasive / All breeds

→ Allantoic fluid does not develop until the 6th day of incubation

The French case

PPILOW The French decree

The French decree 2022-137 of February 5, 2022 prohibits the killing of chicks of *Gallus gallus* breeds reared for table eggs production.

Out of the scope of the decree application:

-other bird species (ducks for example)
-chicks used by the pharmaceutical industry (the vaccines produced against human influenza require the use of fertilized eggs) or for veterinary diagnostics,
-chicks hatched for animal feed purpose,
-chicks that are injured or having a disease likely to cause suffering,
-hatched male chicks resulting from sexing errors,
-chicks from white breeds (for which *in ovo* sexing was lacking at the time of the decree publication)

The decree also stipulates that:

-the equipment installed in hatcheries cannot be questionable for a period of 5 years
-the selected methods need to be applicable for fertilized eggs ≤ 13 days of incubation

PPILOW The French choice: only non invasive techniques

		Agri Advanced Technology (Germany)	Orbem (Germany)	Nectra (France)	Orvia/Nectra (France)	Grimaud frères (France)
Targeted species		*G. gallus*	*G. gallus*	*G. gallus*	*A. platyrhynchos & Cairina moschata*	*A. platyrhynchos & Cairina moschata*
Day of detection		13	12-13	13.5	8.5-9	9-10
Specificities	**Precision**	96%	>95%	>95%	95%	95%
	Breeds	Brown breeds sexable on feather colour	All breeds	Brown breeds sexable on feather colour	Breeds sexable on eye colour	Breeds sexable on eye colour
	Speed	18-20 000 eggs/h/machine	3000 eggs/h/machine but possibility to have several machines on parallel	20 000 eggs/h/machine	20 000 eggs/h/machine	*Confidential*
Hatchability		92.5%	Not communicated	No impact	No impact	90-95% (to be confirmed on industrial level)
Objectives		To improve precision, speed, early detection	To improve precision, speed, early detection	To improve precision, speed, early detection	To improve precision, speed, early detection	To improve speed (industrialisation in progress)
French hatchery		Lohman, Hy-line	Lanckriet, Hendrix Genetics	Novoponte	Orvia	Grimaud frères sélection

PPILOW Fate of sorted eggs (European legislation)

Hatchery by-products

- stillborn and killed chicks,
- Eggshells from hatched chicks
- ***unhatched incubated eggs, including eggs sorted after in ovo sexing***
- clear (unfertilized) eggs
- stored (but not incubated) eggs

= several tons of solid waste, the recovery of which is subject to government and health regulations

According to European regulations (EC n° 1774/2002 of October 3, 2002), clear eggs, incubated eggs and certain other by-products (shells, cuticles, juice, chicks dead after hatching, chicks that have received veterinary treatment or slaughtered for zoonosis control purposes) are classified in **category 2**

-Low risk to public health,
-Can be used as organic fertilizers, for composting, or conversion into biogas

But direct use of these by-products on soil and in compost or biogas production, without prior pressure sterilization, **is strictly forbidden**

PPILOW Fate of sorted eggs (European legislation)

Some hatched chicks may fall into category 3 (particularly if they have been slaughtered because they could not be used in the human food chain= male chicks of layer strains)

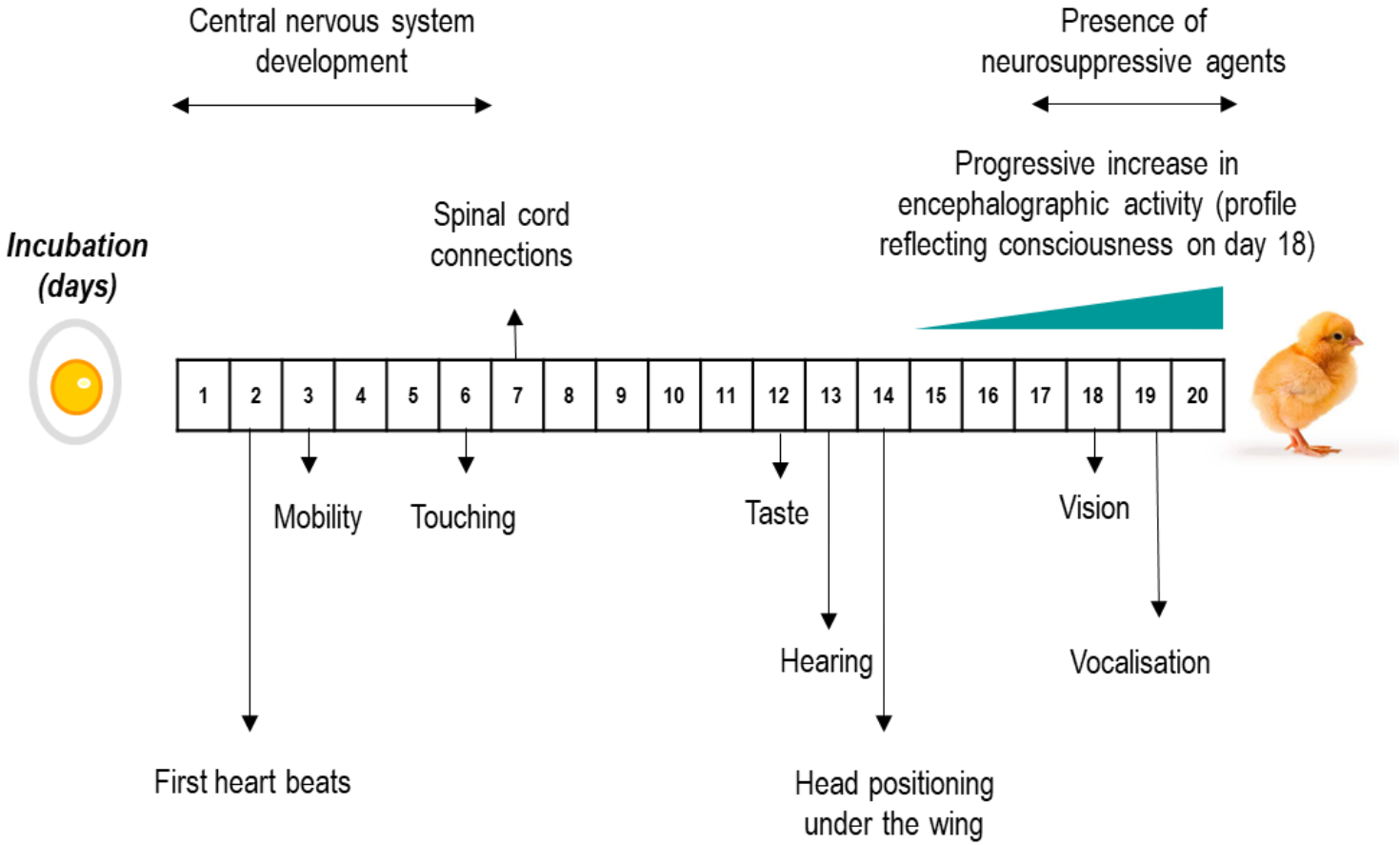
May be used for animal feed (after carbon dioxide inhalation and freezing) : protected animal species, animals hosted in zoos, parks or wildlife protection associations, or even certain privately-owned animals

= reptiles and birds of prey

This derogation related to the French decree 2022-137 of February 5, 2022 is included in the decree of November 4th, 2022, and concerns in particular any chick whose embryo cannot be sexed by the colour of its feathers

The question of embryo suffering and the necessity to develop early *in ovo* sexing methods

PPILOW The question of the onset of the ability to feel pain remains controversial



Controversy depending on the parameters/indicators that are used
Absence of consciousness until day 15 of incubation
Grey zone between 8 and 13 days

The question of when the embryo acquires the ability to feel pain remains essential to increase the acceptability of ovosexing methods

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC)

Effective from 01/02/21

https://policy.brown.edu/policy/avian-embryo-use

AVMA American Veterinary Medical Association

Management of sorted male embryos

Embryos more than 80% developed (>16 days for chicken embryos) = live animals

Approved euthanasia techniques

-cooling at 4°C or -20°C for 4 hours

-freezing of embryos <16 days and CO_2

-anesthetic overdose or decapitation, CO_2 (70% for 5 minutes to 90% for 20 minutes) for embryos or >16 days old

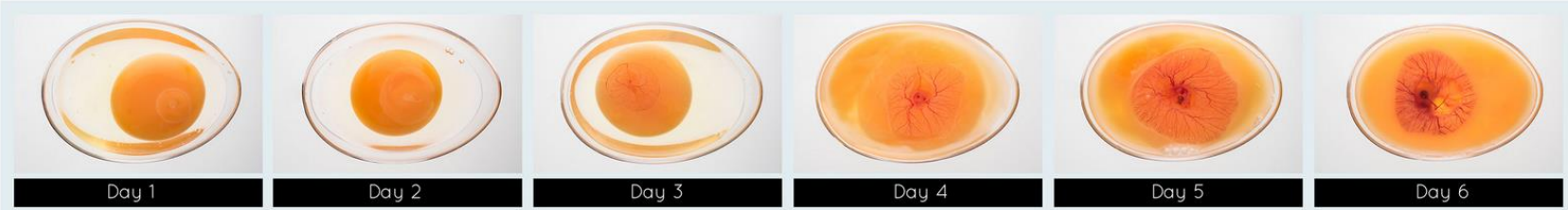
Other developments

Methods that are non invasive and efficient at early stages are likely to be preferred over other ones
The earlier, the better

WP5.3 Development of new ovosexing methods (1/2)

Subtask 1: identification of early biomarkers of sex in ovo (≤ 8 days)

Invasive: collection of the different structures of the egg; identification of the sex of the embryo (from embryonic or extra-embryonic structures)



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Objectives:
-to provide a list of sexual dimorphic molecules (genes, proteins, lipids, metabolites and hormones...)
-to identify which egg structure is the most different between male and female-containing eggs and which structure is the more relevant to focus on

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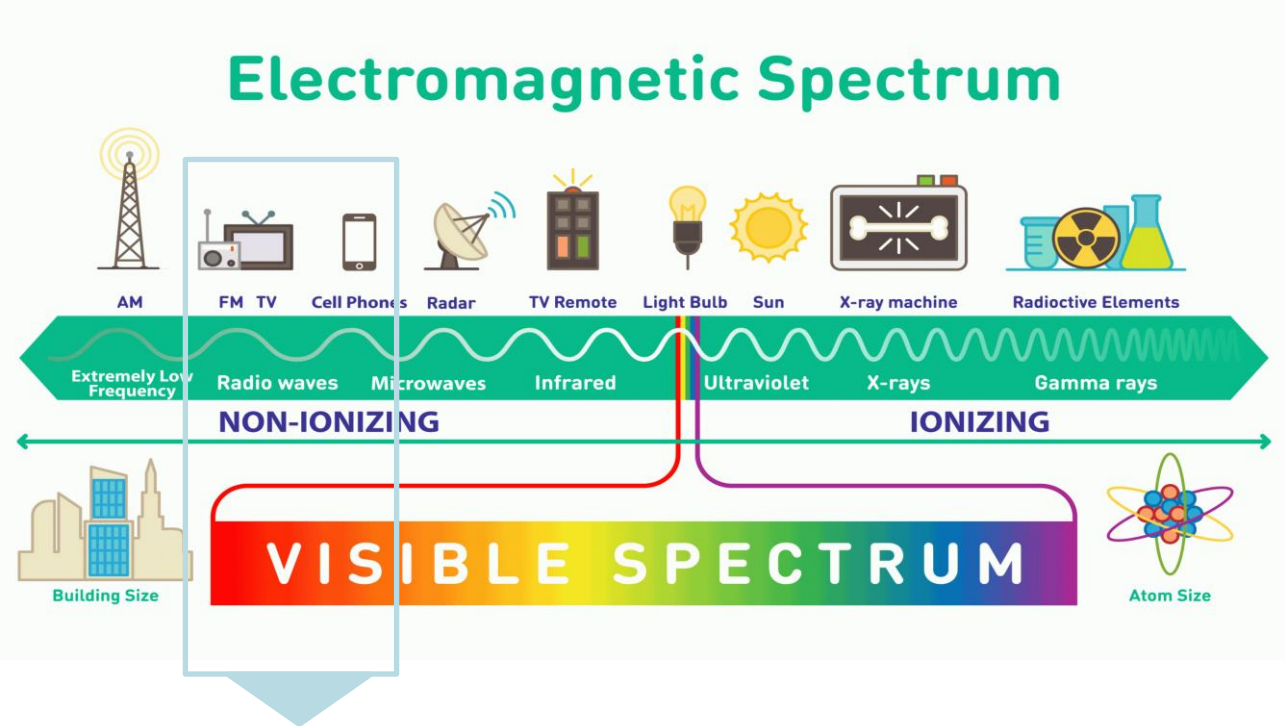
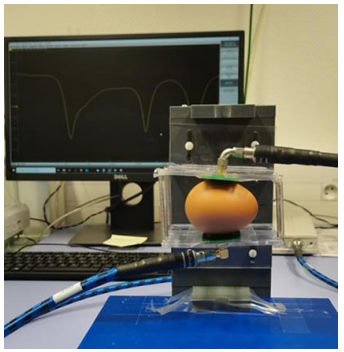
WP5.3 Development of new ovosexing methods (2/2)

Subtask 2: Development of a non-invasive ovosexing technique using radiofrequency (RF)





-frequency range = non-ionising, not harmful
-expected to be of low cost

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Sex-reversal skewed in favour of ♀

SOOS developed an incubation system that affect the sex development process in poultry embryo and turn genetic males into functional female chicks. Our system operate an incubation protocol that control a combination of:

Temperature | Humidity | CO2 levels | Sound Vibration

« During incubation, we influence the expression of genes responsible (DMRT1) for the development of the reproductive system...(to) transform male embryos into egg laying females »

Gene editing : CRISPR/CAS9

eggXYt

CSIRO

Sexing before incubation
but what is the acceptability of a GMO-derived product?
+ dependence on "edited" strains...

PPILOW Take-home messages

In ovo sexing methods are based on
-Z and W sex chromosome differences (D0)
-Anatomical differences (gonad development) (D6)
-Phenotypic differences (feather colour, eye colour, etc.) (D12)
-Molecular differences (D0?)
-Metabolic differences (nutrient utilization kinetics) (D0?)

♂ / ♀ differences appear at specific stages of development, which may limit the possibilities of using certain ovosexing approaches at earlier stages (since differences are not visible)

Several in ovo sexing studies/developments/improvements are in progress

The two other alternatives are still investigated (rearing brothers of laying hens, development of dual-purpose crossbreeds)

Review will be published in December in a French journal
English version available in OA during the first semester 2024

LA REVUE INRAE PRODUCTIONS ANIMALES

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Thank you for your attention

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