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KARINA PEREIRA LOPES

O USO DE EMBRIÕES COMO MODELOS EXPERIMENTAIS

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Trabalho de Conclusão de Curso apresentado ao Curso de Biomedicina da Universidade Federal de Uberlândia como requisito para a obtenção do Título de Bacharel em Biomedicina.

Orientador: Prof. Dr. Carlos Ueira Vieira

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1 Review

2 The use of embryos as experimental models

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11 **Abstract:** Animal embryos are vital tools in scientific research, providing insights into biological
12 processes and disease mechanisms. This paper explores their historical and contemporary
13 significance, emphasizing the shift towards refining *in vitro* systems as alternatives to animal
14 experimentation. Across various species like rodents, rabbits, fish, amphibians, birds, and reptiles,
15 different experimental models play crucial roles in understanding embryonic development and
16 physiological processes. We conducted a data survey in relevant literature on the use of embryos
17 in research and combined the data with the aim of presenting the importance of this model for
18 scientific advancements and the ethical considerations and regulations surrounding embryo
19 research, highlighting the importance of minimizing animal suffering while promoting scientific
20 progress through the principles of replacement, reduction, and refinement. Mammalian embryos
21 aid in studying embryogenesis and human conditions, while avian embryos are intermediate
22 models for toxicity studies. Amphibian embryos offer insights into gene expression, reptile
23 embryos provide understanding of genetic processes and thermoregulation, and fish embryos are
24 valuable for genetics and ecotoxicity research. In conclusion, animal embryos facilitate
25 transformative advancements in science and medicine, necessitating adherence to ethical
26 principles and legal frameworks to uphold animal welfare and ensure responsible research
27 conduct.

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Keywords: Animal embryos; biomedical research; 3Rs; animal ethics; alternatives methods

1. Introduction

Animal models have played a pivotal role in driving the most significant
advancements in numerous biological domains, enhancing our understanding of
anatomy, physiology, pathology, and pharmacology [1].

For exemplo rodents are extensively used in various areas of human medical
research, such as surgery, transplantation, cancer, diabetes, psychiatric disorders,
neural regeneration, wound and bone healing, space motion sickness, and
cardiovascular disease. They also play a crucial role in drug development,

39 demonstrating therapeutic efficacy and assessing the toxicity of new compounds before
40 human trials [2].

41 Pigs, exhibit comparable anatomical size and structure, immunology, genome, and
42 physiology to humans, making them a preferred choice over rodent models for
43 translational and clinical research purposes [3]. Due to the substantial resemblance
44 between pig and human genome sequences, pigs are regarded as invaluable models for
45 exploring human health [4]. Given their anatomical resemblance to humans it is
46 extensively utilized for research especially in craniofacial and ocular studies [3].
47 Furthermore, pigs fulfill a crucial role in biomedical research as organ and tissue
48 donors, addressing the scarcity of transplant materials for humans. Their analogous
49 organ structure and function position pigs as promising candidates for
50 xenotransplantation, offering potential remedies for various transplant procedures [4].

51 Another illustration can be Zebrafish, which is increasingly recognized as a
52 valuable tool for modeling human diseases, demonstrate the ability to replicate diverse
53 aspects of human physiology and pathologies, with approximately 70% of human
54 genes finding clear counterparts in their genome [5]. Noteworthy contributions in
55 cancer research, wherein zebrafish serve as models for studying cancer development
56 and as platforms for drug testing, underscore their growing significance in advancing
57 medical science [5].

58 Animal models facilitate the investigation of molecular and cellular functions,
59 including intricate processes such as circulation, hormone regulation, cellular
60 structures, and tissue functions, under both normal and pathological conditions [6].
61 Additionally the involvement of animals in biomedical research has played a critical
62 role in progressing our comprehension of diseases, devising treatments, and assessing
63 novel therapies, while also making substantial contributions to the creation of new
64 drugs and vaccines, along with pioneering surgical techniques and anesthesia protocols
65 [7, 8].

66 In recent years, there has been a significant surge in scientific attention towards
67 enhancing the physiological accuracy of *in vitro* systems and extending their versatility
68 to advance the goals of replacing, reducing, and refining (3Rs) animal experimentation
69 [9].

70 Currently, there are several types of alternative methods, also known as
71 non-animal methods [10]. For example, *in silico* techniques (computer models and
72 simulations) utilize computer software, often incorporating mathematical equations, to
73 simulate real-world processes such as creating the structure, function, and metabolism
74 of human body organs [11]. Another example is the use of animal body parts as an
75 alternative to the Draize eye irritation test, which involves live rabbits. The Bovine
76 Corneal Opacity and Permeability assay (BCOP) utilizes eyes obtained from animals
77 slaughtered for consumption [10]. Cell and tissue cultures are also employed as
78 alternative methods. Additionally, there's the Organ-on-a-Chip technology, which
79 mimics *in vivo* conditions, providing opportunities for deeper insights into the
80 development of numerous human diseases and offering an enhanced platform for

81 testing new drug compounds [12]. Additionally, the use of animal embryos is another
82 alternative method [10].

83 For instance, in a study by Zoses *et al.* (2021), fertilized chicken eggs were
84 employed as an alternative model to explore the distribution of two antiepileptic drugs,
85 during two developmental stages. Their investigation revealed that the drugs injected
86 into the egg were swiftly disseminated to the chicken embryo brain, attaining
87 concentrations comparable to those in the human central nervous system (CNS),
88 suggesting that the developing chicken embryo offers a dependable and suitable model
89 for prenatal pharmaceutical research [13]. Similarly, Tajaki *et al.* (2023) conducted a
90 study focusing on Xenograft of human pluripotent stem cell-derived cardiac lineage
91 cells on zebrafish embryo heart. Fluorescence-labeled cardiac lineage cells, derived
92 from human induced pluripotent stem cells (hiPSCs), were microinjected into the
93 zebrafish heart, observing migration into the pericardial cavity and heart one day
94 post-injection [14]. Notably, some injected cells exhibited heartbeat-like movements
95 within the zebrafish heart, indicating successful xenografting of hiPSC-derived cardiac
96 lineage cells. This innovative approach establishes zebrafish embryos as a valuable
97 model organism for investigating the molecular and cellular mechanisms underlying
98 the grafting process [14].

99 Given what has been discussed, the present work aims to explore the use of
100 embryos as experimental models, highlighting their importance in contemporary
101 scientific research. Throughout this study, the different types of embryos used in
102 research will be investigated, covering a wide range of species from mammals to fish.
103 The ethical and legal aspects related to the use of the animals models will be analyzed,
104 as well as the significant contributions these models have made to understanding
105 developmental biology and discovering new medical therapies.

107 **2. Importance of experimental models for scientific and technological** 108 **development and the need for alternative methods**

109
110 Scientific research plays a crucial role in advancing knowledge, experimental
111 models are essential tools in this process. The term "animal model" comes from the
112 Latin word "*animae*," meaning soul or spirit, indicating that organisms are animated
113 and "model" refers to an object of imitation. Consequently, an animal model is a living
114 representation used to examine physiological and pathological processes [15]. In this
115 context, *in vitro*, *ex vivo* and *in vivo* models emerge as fundamental strategies for
116 understanding complex biological phenomena [16].

117 The *in vitro* model approach plays a significant role in identifying therapeutic
118 targets and screening compounds for drug development, allowing cellular and
119 molecular mechanisms to be investigated with high precision outside the organism [17].
120 Isolated cells or tissues are cultured under conditions that simulate the organism's
121 physiological environment. This method offers several advantages, including greater
122 experimental control, precise manipulation of variables, and reduced complexity

123 associated with more complete biological systems [18]. Emerging technologies such as
124 organoids and tumoroids, represent emerging technologies within the realm of cancer
125 research and drug discovery [19]. Microphysiological systems (MPS), like microfluidic
126 organs-on-chips, utilized to mimic human physiology and disease processes, being a
127 valuable tool across research, development, diagnosis, and treatment, and beneficial
128 especially in initial drug screening and cell therapies [20, 21]. Cell-based
129 high-throughput screening (HTS) is employed to uncover initial compounds for
130 small-molecule drug design [22, 23].

131 *Ex vivo* models, involves studying tissues from organisms under controlled
132 conditions to mimic their natural state, offering advantages such as minimal alteration
133 and the ability to conduct tests not possible in living subjects due to ethical concerns
134 [24]. An illustration can be found in a study conducted by Stampfl et al. (2011), where
135 an isolated beating heart (Langendorff heart) was employed as a model system to
136 investigate the cardiovascular effects of engineered nanoparticles (ENPs), aiming to
137 comprehend the potential cardiovascular risks associated with exposure to these ENPs
138 [25].

139 *In vivo* models, conducted on living organisms and used based on comparative
140 medicine, are considered fundamental for a more comprehensive understanding of
141 complex biological interactions [26]. There is a wide variety in the classes of animals
142 used as experimental models. The correct animal model is essential for the success of
143 research, as each animal has its own specificity. Some examples are: mice, rats, rabbits,
144 avian, fish and reptiles [2].

145 Rodents are widely recognized as the most used experimental models in scientific
146 research, representing approximately 95% of animals manipulated in the laboratory
147 [17]. Annually, worldwide, biomedical research involves the utilization of a minimum
148 of 120 million mice and rats [27-29]. The choice of these animals is justified by several
149 factors, such as: well-known genetic background, allowing greater predictability in
150 experimental results, the three species - rats, mice, and humans - share approximately
151 30,000 genes, with about 95% of these genes being common to all three species, as well
152 as the creation of transgenic animals such as knockouts (KOs) and knockins [2, 30, 31].

153 Rabbits play a crucial role in scientific research due to their greater phylogenetic
154 proximity to humans compared to rodents. The short lifespan and rapid gestation
155 period allow experiments to be carried out over several generations in a short space of
156 time. The size of these animals is also advantageous as it allows a variety of analyzes to
157 be performed on a single animal [32].

158 In relation to fish, the zebrafish, known scientifically as *Danio rerio*, stands out as a
159 research model as it has genetic homology with man. Thus, with the possibility of
160 genetic changes and dietary changes, studies related to metabolic disorders such as
161 obesity can be carried out with these animals [33]. When considering amphibians,
162 salamanders and frogs emerge as prominent experimental models frequently
163 employed in research, particularly concerning studies elucidating embryonic
164 development and physiological adaptations [34].

165 The quantity of animals utilized in research has risen alongside the progress in
166 medical technology research and development [35]. Recently, scientists have widely
167 recognized that animals are also capable of experiencing pain and distress [36]. Hence,
168 ethical dilemmas emerge from the potential harm and suffering animals endure during
169 experiments, leading to inquiries into the moral justifications for such actions [8].
170 Consequently, scientific organizations and government regulatory agencies are
171 increasingly recognizing the potential of alternative methods to replace animal testing.
172 This shift could lead to improved efficiency and safety in the development of new
173 therapeutics for human use [37].

174 An alternative or non-animal method refers to techniques or tests that replace,
175 diminish, or enhance traditional animal experimentation by utilizing other testing
176 methods [38]. For instance, utilizing embryos in scientific research demonstrates an
177 alternative to the use of *in vivo* animals when carrying out experiments [39]. An
178 example of this is the utilization of embryonated chicken eggs in studying Pthiosis,
179 suggesting the feasibility of conducting experiments related to fungal infection without
180 resorting to live animal testing [39].

181 Therefore, the use of experimental models is essential for scientific development.
182 The choice of species depends on the objective of the study and must be chosen
183 respecting the physiological individualities of each animal [40].

184 3. Use of embryos in research

185
186
187 The use of embryos as experimental models can be used in various areas of
188 science such as metabolic disorders [41], vascular changes [42] and immunology [43].
189 The focus and objectives of the experiment determine the choice of the animal that will
190 give rise to the embryo [17]. These species range from rodents to non-human primates
191 [44]. In the class of mammals, mice and rabbits are frequently utilized in embryo
192 culture studies [45, 46].

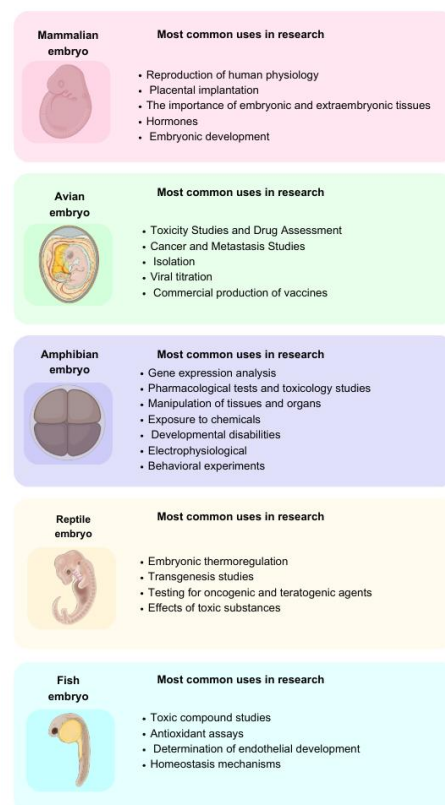
193 Avian embryos, particularly chickens, are commonly subjected to research
194 methods such as the "*in ovo*" technique, in order to examine morphological changes
195 during embryonic development within the eggshell and offer valuable insights into
196 various physiological processes (Figure 1)[47-49]. Lehel et al. (2021), investigated the
197 effects of pesticides, including copper sulfate and glyphosate, on chicken embryos.
198 Exposure to copper sulfate during incubation resulted in increased mortality and
199 decreased body mass, while glyphosate exposure led to higher mortality rates and
200 abnormalities. Glyphosate-based herbicides also impacted plumage and organ
201 development. Interactions with surfactants such as phenoxyethanol exacerbated
202 toxicity, and combined exposure with heavy metals intensified mortality and
203 abnormalities [50]. In a study by Ruth et al. (2008), explored the impact of iron
204 complexes on avian embryos, focusing on differences in toxicity profiles among
205 various compounds. FeD (iron dextran) showed the highest tolerance, with no
206 adverse effects on survival or embryo weight, whereas FeG (iron gluconate) exhibited

207 the most toxicity. Analysis revealed significant increases in liver and kidney iron
208 levels across all complexes, particularly with FeG. Variances in iron absorption were
209 observed between injection methods. The study underscores the importance of
210 considering administration route and iron complex type when assessing their impact
211 on avian embryos. Additionally, distinct toxicity profiles were observed between *in*
212 *ovo* and *in vitro* experiments [51].

213 Reptile embryos, especially those of turtles, are frequently employed in research
214 investigating embryo thermoregulation and its implications for embryonic
215 development in response to climate change [52, 53]. Temperature directly influences
216 the metabolic and developmental rates of embryos, potentially resulting in long-lasting
217 effects on phenotype that endure beyond the embryonic stage [52]. Ye et al. (2021),
218 investigated Chinese three-keeled pond turtle embryos, uncovering the roles of
219 MrTRPA1 (transient receptor potential ankyrin 1) and MrTRPV1 (transient receptor
220 potential vanilloid-1) as thermal sensors. These TRP (thermo-transient receptor
221 potential) channels collaborate to guide embryos toward optimal temperatures and
222 away from harmful heat, elucidating mechanisms of thermotaxic behavior. While
223 TRPV1 detects high temperatures, TRPA1 appears specialized for warmth detection in
224 reptiles, suggesting evolutionary adaptations in thermal sensing across species. The
225 distribution of these channels in sensory neurons suggests distinct pathways for
226 temperature recognition, potentially influencing reptilian thermoregulation strategies
227 [54] (Table 4).

228 Amphibian embryos have also been prominent in research endeavors,
229 contributing to the understanding of physiological and pathological effects of various
230 agents, like putative toxins and teratogens on its development [55]. As an exemple, the
231 study conducted by Viriato et al (2021) , assessed the teratogenic and toxic effects of
232 herbicide 2,4-D (DMA® 806) on bullfrog embryos and tadpoles. Results showed
233 minimal teratogenicity but low toxicity on embryos and tadpoles, with potential
234 growth inhibition at lower concentrations. Tadpoles exhibited inflammatory responses,
235 erythrocytosis, and organ injuries, suggesting physiological stress and dehydration due
236 to pesticide exposure [56]. Futhermore, some of amphibian species have the remarkable
237 ability to regenerate limbs which make them highly sought after for transgenic and
238 knockout technology [55]. In a study spearheaded by Khan and Crawford (2020), the
239 comparison between larval and adult limb blastemas in *N. viridescens* revealed distinct
240 morphological features and developmental trajectories. While larval blastemas
241 exhibited preaxial dominance and sequential digit emergence characteristic of typical
242 urodele forelimb development, adult blastemas showed simultaneous digit emergence
243 and lacked preaxial dominance, mirroring embryonic patterns seen in mice. These
244 findings suggest that larval limb regeneration in *N. viridescens* involves unique
245 regulatory mechanisms rather than a simple recapitulation of embryonic development.
246 Moreover, the similarities between adult newt regenerates and amniote development
247 imply an evolutionarily derived preaxial dominance [57].

248 In regard to small fish model, the prominent species involved in research are
249 zebrafish [58, 59] and to a lesser extent killifish species (cyprinodontiform), such as the
250 Japanese Medaka [58, 59]. Zebrafish embryos and larvae have emerged as valuable
251 alternative animal models for toxicity testing purposes [60]. This shift towards
252 zebrafish models is driven by their numerous advantages, including their rapid
253 development, transparent nature allowing for easy observation of internal structures,
254 and genetic similarity to humans [58, 61, 62]. As an example Sun et al. (2021) conducted
255 a study on the cardiovascular effects of nanoplastics using zebrafish embryos, revealing
256 dose-dependent impacts like pericardial edema and yolk sac degeneration, suggesting
257 direct interference with cardiovascular development. Nanoplastics accumulated in
258 crucial areas, disrupting heart rate and vascular formation. Additionally, they induced
259 hypercoagulability and increased thrombosis incidence, potentially via endothelial
260 dysfunction and altered blood flow. Oxidative stress and inflammation were
261 implicated, with nanoplastics triggering ROS generation and systemic inflammatory
262 responses [63].
263



264
265 **Figure 1.** Animal embryos and their most common uses in research.
266

267 With most of animal embryo species used in research mentioned above, the
268 legislation in force for such research type in main countries will be discussed from now
269 on.
270

4. Ethical and Legal aspects related to the use of animals as experimental models

The use of animals in research plays a crucial role in the advancement of scientific knowledge and the development of medical treatments, but it also raises important ethical and moral issues [64]. Therefore, countries around the world have developed laws and regulations to govern and supervise the use of animals in scientific experimentation, with the aim of ensuring that animal suffering is minimized, while at the same time promoting the pursuit of scientific progress [64, 65].

The laws and regulations cover a variety of aspects, including the acquisition and care of animals, permitted experimental procedures, ethical review of research protocols and monitoring of facilities where research is conducted [66].

One of the fundamental pillars of the regulation is the principle of the 3Rs: replacement, reduction, and refinement [67]. Replacement refers to the search for alternative methods or technologies that avoid the use of animals whenever possible, such as computer models, cell cultures, bioengineering techniques, among other approaches. It may also include the use of animals that are not sensitive to suffering, such as invertebrates and immature forms of vertebrates [68].

Reduction aims to minimize the number of animals used per experiment, ensuring that studies are planned efficiently to obtain robust and reproducible results, as well as maximizing the information obtained from each animal to reduce the need for additional animals, data and resources sharing among research groups and organizations contributes to these reduction efforts [69].

In turn, refinement seeks to improve experimental procedures to reduce suffering and improve the welfare of the animals involved, providing adequate housing conditions, the use of anesthesia and analgesia, and training to reduce stress. These practices not only improve animal welfare, but also increase the reliability of research results [68].

The following are some legal instruments for controlling the use of animals for scientific purposes around the world for a comparative analysis.

4.1 European Union

In Europe there are two legal instruments in force for the use of animals for scientific purposes, namely the European Convention for the Protection of Vertebrate Animals in Research (ETS 123) [70] and Directive 2010/63/EU of the European Union [71].

ETS 123 establishes minimum guidelines for the ethical use of vertebrate animals in research, teaching and testing covering a variety of species, including mammals, birds, reptiles and amphibians. This convention is based on some essential principles, such as the promotion of the 3Rs, animal welfare (Article 5), wherein researchers must ensure that the animals used receive proper care and are respected throughout the

313 research process so that the physical and mental health of these animals is preserved.
314 This implies providing adequate housing conditions, balanced nutrition, access to
315 veterinary care and environmental stimulation whenever feasible [70].

316 It stipulates that experiments should be conducted in an ethical manner,
317 minimizing animal suffering and justifying their use by significant scientific benefits,
318 and whenever possible alternative measures providing the same results should be
319 implemented (Articles 6 - 8). To ensure that experiments are conducted responsibly
320 and in accordance with established guidelines, prior authorization by the competent
321 authorities and supervision by qualified professionals is required (Articles 13 and 25).
322 Therefore, it is extremely important to provide adequate education and training to
323 professionals involved in animal use, ensuring ethical and appropriate animal handling
324 (Article 26) [70].

325 Furthermore, it mandates that information on how animals are used in research be
326 shared openly and transparently (Articles 27 and 28). By encouraging the disclosure of
327 statistics on animal use and advancements in alternative methods, it promotes a
328 transparent environment enabling open discussions about animal research practices
329 [70].

330 Directive 2010/63/EU, issued by the European Parliament and the European
331 Council, is a European Union (EU) regulation that defines guidelines for the protection
332 of animals used in scientific experiments in EU member states. This directive replaces
333 Directive 86/609/EEC and its main objective is to standardize the regulation, training,
334 housing, animal care, restrictions on the use of certain animal species, review and
335 authorization of projects throughout the EU to ensure the protection of animals used in
336 experiments, specifically non-human vertebrate animals and some invertebrates
337 capable of feeling pain. In addition, it explicitly addresses the 3Rs in its Article 4 [71].

338 The directive also emphasizes the necessity of ethical standards in research
339 utilizing embryonic models of mammals, highlighting the risks of pain, suffering, and
340 distress, especially during crucial developmental stages. This stance is supported by
341 scientific findings revealing that embryonic and foetal forms, particularly as they
342 progress into later developmental phases, are more susceptible to these adverse effects.
343 This vulnerability is further pronounced if these developmental stages extend beyond
344 the initial two-thirds of their developmental period, potentially leading to negative
345 impacts on their well-being and subsequent development [72].

346 Although ETS 123 and Directive 2010/63/EU share similar objectives of animal
347 protection and regulation of animal use in experiments, they operate in distinct legal
348 spheres. Whereas ETS 123 is an international treaty established by the Council of
349 Europe, Directive 2010/63/EU is European Union legislation applicable to EU member
350 states [70, 71].

351 4.2 North America

352 In North America, both Canada and the United States boast well-established
353 laboratory animal programs that have evolved from a variety of laws, rules, guidelines,
354

355 and procedures over time [65].

357 4.2.1 *United States of America*

358 In 1966, the Animal Welfare Act (AWA) was introduced, which establishes
359 standards of care, including housing, feeding, and veterinary care, to ensure a healthy
360 and suffering-free life for animals. Facilities housing animals must obtain licenses to
361 operate legally and are subject to regular inspections by the U.S. Department of
362 Agriculture to promulgate regulations and enforce non-compliance [73]. Additionally,
363 the act regulates the trade of animals, preventing illegal trading and ensuring proper
364 care during sale. For animals used in research, additional requirements are established
365 to ensure ethics and minimize suffering, with oversight from research ethics
366 committees[73]. This legislation covers warm-blooded vertebrate animals used for
367 research, with exceptions for some such as birds, rats of the genus *Rattus* and mice of
368 the genus *Mus*, which are bred for the purpose of being used in scientific studies;
369 horses which are not used for research purposes; among others [73].

370 The second law is the Health Research Extension Act (HREA) of 1985, which is an
371 amendment to the Public Health Service Act and applies to institutions that receive
372 funding from the US Public Health Service [74]. One of the most notable features of this
373 legislation is the obligation imposed on all funded research institutions to establish
374 animal care committees. These committees, known as Institutional Animal Care and
375 Use Committees (IACUCs), are responsible for ensuring that all research carried out at
376 the institution complies with the guidelines established by the law. The IACUCs,
377 composed of a minimum three members, including a veterinarian and an individual
378 not affiliated with the institution, are charged with carrying out periodic reviews of the
379 care and treatment provided to animals in research facilities, ensuring that they comply
380 with the stipulated animal care standards [74].

381 In addition, the legislation stipulates that institutions must provide training in
382 humane animal care and experimentation practices for scientists, animal technicians
383 and others involved in the care, treatment and use of animals in research. This includes
384 the proper use of medications such as tranquilizers, analgesics and anesthetics, as well
385 as methods to reduce the animals' discomfort and pain during research procedures [74].
386 In situations where the conditions of care, treatment or use of animals do not comply
387 with the guidelines established by the law and corrective measures are not
388 implemented by the research institution, the legislation provides for the possibility of
389 suspension or revocation of research grants or contracts [74].

390 An extra framework outlined in the "Institutional Animal Care and Use
391 Committee Guidebook," available in the IACUC Central, researchers, in order to work
392 with animal embryos, need to clearly specify the quantity of fertilized one-cell eggs,
393 embryos, or fetuses required for their proposed studies-This entails detailing the
394 estimated number of experimental animals needed, which may be restricted to the
395 number of female animals undergoing specific procedures such as mating, euthanasia,
396 or surgical manipulation for the collection of eggs, embryos, or fetuses [75].

4.2.2 Canada

The Canadian Council on Animal Care (CCAC) is an essential entity for ensuring the welfare of animals used in research, education, and scientific testing in Canada. Founded in 1968 as a standing committee of the Association of Universities and Colleges of Canada, the CCAC was established following recommendations made in a report by the Special Committee on the Care of Experimental Animals in 1966. This committee proposed the creation of a voluntary control program, where scientists at each institution would commit to implementing the guiding principles of an independent advisory body, subject to peer review [76].

Prior to the creation of the CCAC, guidelines for the care and use of experimental animals in Canada were scarce, limited to the 1961 Guiding Principles on the Care of Experimental Animals of the Canadian Federation of Biological Societies (CFBS). The CCAC played a pioneering role in the creation of local animal care committees, responsible for the ethical use of animals in their institutions. These local committees are responsible for ensuring the ethical use of animals and adherence to the guidelines established by the CCAC at a local level. Before any study begins, they must evaluate the ethical aspects of the proposed research [76].

In addition to setting standards and guidelines, CCAC offers certification and accreditation programs for institutions and researchers, ensuring compliance with the highest ethical and animal care standards. Additionally, it provides educational resources and training for professionals involved in the use of animals in research, promoting awareness of ethical issues and best animal care practices [76].

A crucial function of CCAC is animal rights advocacy, working in collaboration with other organizations and government agencies to promote policies and regulations that protect the welfare of animals used in research. In short, CCAC plays an integral role in promoting ethics and quality in the use of animals in scientific and educational research, while ensuring their welfare and protection [77, 78].

4.3 Africa

In Africa, only five countries - Kenya, Tanzania, Seychelles, Zimbabwe and South Africa - have legislation dedicated to regulating the use of animals in scientific research [79].

4.3.1 Tanzania

In Tanzania, the Animal Welfare Act of 2008 applies specifically to the Tanzanian mainland, excluding animals governed by the Fisheries Act and the Wildlife Conservation Act [80]. One of the most significant features of this law is the clear definition of the term "animal", covering both vertebrates and invertebrates, except humans. This extends legal protection to a wide variety of species, recognizing their importance and sensitivity as living beings [80].

In the context of animal experimentation, the law is based on the principles of the

439 3Rs and establishes fundamental principles of animal welfare, which reflect a
440 commitment to the proper and compassionate treatment of animals. These principles
441 include the five freedoms, ranging from the guarantee of food and water to the
442 freedom to express natural behaviors. Furthermore, the legislation recognizes animal
443 welfare as crucial to the development of a morally and culturally advanced society,
444 highlighting the legal and moral responsibility of human beings to care for and protect
445 animals [80].

446 In its 5th Part, the legislation establishes a series of fundamental guidelines to
447 guarantee the welfare and ethical treatment of animals in surgical procedures,
448 biotechnology and animal experimentation. The law establishes that surgeries can only
449 be performed for the purpose of healing, protecting animals from procedures aimed
450 solely at modifying their appearance [80].

451 Regarding biotechnology and genetic manipulation, any genetic modification in
452 animals must be authorized by the competent Ministry, ensuring that the procedures
453 comply with ethical and scientific standards [80]. Authorization for animal
454 experimentation is another important measure, ensuring that experiments are only
455 conducted when there is potential to benefit human or animal health, following
456 rigorous ethical and animal welfare criteria. These experiments must be conducted by
457 authorized individuals, who are responsible for ensuring that they are carried out
458 ethically and minimizing the suffering of the animals involved. Holders of permits for
459 experiments are required to maintain detailed records and provide veterinary
460 supervision of the animals involved, ensuring their welfare [80].

461 4.3.2 Kenya

462 The Prevention of Cruelty to Animals Act was enacted in 1962 and revised in 2012.
463 Initially, the law stipulates that only licensed individuals are allowed to carry out
464 experiments on animals and any individual who conducts experiments without such a
465 license is subject to penalties, including fines and imprisonment [81].

466 Additionally, the legislation prohibits experiments from being carried out under
467 certain specific conditions, such as when they are not in accordance with the terms of
468 the licensee's license or when they are intended to acquire manual skills or illustrate
469 lectures without proper authorization [81].

470 The restrictions on licensees conducting experiments are strict. Experiments can
471 only be carried out to promote new physiological discoveries, save lives, alleviate
472 suffering or fight disease, and only under the written order of a Judge in criminal cases
473 [81].

474 Furthermore, there are strict requirements for animal handling during
475 experiments. The animals must be given anesthesia powerful enough to prevent pain
476 during the entire procedure, and if there is a possibility of pain persisting after
477 anesthesia, the animal must be sacrificed before recovering [81].

478 Licenses for conducting experiments are granted by the Minister and are subject to
479 specific conditions, including the need to carry out the experiments in designated
480

481 locations. Those who are not registered under the Veterinary Surgeons or Doctors and
482 Dentists Acts must carry out the experiments under proper supervision [81].

483 Finally, the legislation also addresses special permits for teaching and revocation
484 of licenses or permits in necessary cases. Regular inspections are allowed to ensure
485 compliance with the law, and the public display of experiments is strictly prohibited,
486 subject to severe sanctions [81].

487 These legal provisions aim to ensure that animal experiments are conducted
488 ethically, minimizing animal suffering and promoting responsible scientific research.

490 *4.4 Latin America*

491 *4.4.1 Mexico*

492 The Mexican Official Standard (NOM) 062-ZOO-1999 is a technical standard
493 issued by Mexico's Secretariat of Agriculture, Livestock, Rural Development, Fisheries
494 and Food (SAGARPA), which aims to guarantee the welfare of these animals, as well as
495 promote uniformity and quality in the procedures related to their handling in scientific
496 research activities. It is applicable to a variety of animals frequently used in
497 experimentation, including rodents, lagomorphs, carnivores, non-human primates and
498 pigs [82].

499 The standard establishes that compliance is mandatory throughout the country,
500 covering all entities, whether individuals or institutions, involved in any aspect
501 involving laboratory animals. Both the Secretariat of Agriculture, Livestock and Rural
502 Development and the state and Federal District governments are responsible for
503 ensuring compliance with these regulations in their respective geographical areas [82].

504 In addition, when an institution uses animals for research, the establishment of an
505 Institutional Internal Committee for the Care and Use of Laboratory Animals is
506 required, whose main objective is to ensure that the care and use of animals in research,
507 testing and teaching contexts are conducted appropriately and humanely. Its
508 responsibilities include drawing up annual reports on the state of care and use of
509 animals, evaluating and approving research protocols, stopping non-compliant
510 procedures, resolving unforeseen issues and other tasks as necessary [82].

511 *4.4.2 Brazil*

512 The Arouca Law, officially named Law N°. 11.794/2008, represents a significant
513 milestone in the regulation of the use of animals for scientific purposes in Brazil. By
514 regulating item VII of § 1 of art. 225 of the Federal Constitution and repealing Law N°.
515 6.638/1979, this legislation establishes essential guidelines and procedures to guarantee
516 the ethics and welfare of animals involved in scientific research and teaching activities
517 [83].

518 One of the key aspects addressed by this legislation is the definition of strict
519 criteria for the breeding and use of animals in higher education institutions and
520 mid-level technical institutions in the biomedical field. Moreover, the law determines
521
522

523 the types of animals covered and stipulates fundamental concepts, such as experiments
524 and humane euthanasia. Additionally, it establishes the creation of the National
525 Council for the Control of Animal Experimentation (CONCEA) and the Ethics
526 Committees for the Use of Animals (CEUAs) [83].

527 CONCEA, in turn, is responsible for formulating and ensuring compliance with
528 the regulations related to the use of animals in scientific activities, including the
529 accreditation of institutions and the evaluation of alternative techniques. CEUAs, on
530 the other hand, are responsible for examining procedures, maintaining updated
531 records, and issuing certificates, contributing to ensuring compliance with established
532 ethical guidelines [83].

533 Furthermore, the Arouca Law regulates in detail the conditions for breeding and
534 using animals for scientific purposes, establishing criteria for procedures, euthanasia,
535 sedation, minimum number of animals, duration of experiments and emphasizes the
536 use of the 3Rs [83]. Given that the 3Rs principle should be prioritized, animal embryo
537 research is permitted if approved by the "Animal Care and Use Committee"
538 (CONCEA), particularly within the context of embryonic transfer from one rodent to
539 another [84]. Techniques involving embryos, such as cryopreservation, are aimed at
540 reducing the production of specific strains, preserving genetic heritage, and
541 minimizing the number of animals housed in research colonies [84].

542 To ensure compliance with these provisions, administrative penalties are provided
543 for institutions and individuals who violate the law, including warnings, fines and
544 temporary or permanent closures [83].

545 By establishing ethical and humane standards, the Arouca Law promotes a
546 responsible and conscientious approach to the use of animals for scientific purposes,
547 contributing to a more ethical and sustainable practice in the field of biomedical and
548 scientific research in general [83].

549 *4.5 United Kingdom*

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551
552 Concerning the United Kingdom, according to the "Guidance on the operation of
553 the Animals (Scientific Procedures) Act (1986), embryonic and fetal forms of mammals,
554 birds and reptiles are considered "protected animals" once they reach the last third of
555 their gestation or incubation period. Procedures conducted on embryonic, fetal, or
556 larval forms of protected animals are regulated if these procedures may cause pain,
557 suffering, distress, or lasting harm beyond a certain threshold, even if the animal has
558 not yet reached the stage of development where it is considered a protected animal,
559 ensuring that ethical considerations are upheld throughout all stages of development
560 [85].

561 Projects involving the utilization of admixed embryos categorized as Category 3 in
562 the AMS report on ACHM (Animal-Human Chimera Models) and Category 2 when
563 the predominance of an admixed embryo is unclear or uncertain entail research
564 endeavors where embryos possess a combination of genetic material from different

565 species, potentially including human and animal components. These categories
566 delineate specific classifications within the regulatory framework, aiming to address
567 ethical and scientific considerations regarding the creation and utilization of such
568 embryos in research contexts [85].

570 4.6 Australia

571
572 In Australia, regulations pertaining to the use of animal embryos are outlined in
573 the Australian Code for the Care and Use of Animals for Scientific Purposes, 8th
574 Edition 2013 (updated 2021). This code ensures ethical and humane treatment
575 throughout research endeavors involving embryos. When a project involves the fetus
576 or embryo, specific considerations are mandated. Firstly, the requirements for
577 anesthesia and analgesia must be carefully addressed to mitigate any potential
578 discomfort or pain experienced by the fetus or embryo during procedures, as detailed
579 in Clauses 3.3.8–3.3.15 [86] (updated 2021).

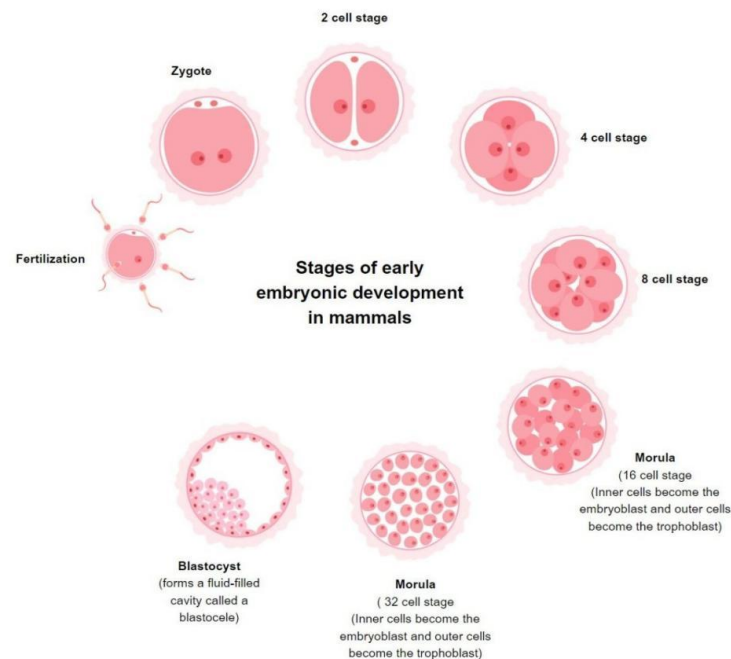
580 Furthermore, if a procedure conducted on a fetus or embryo is anticipated to
581 compromise the animal's ability to survive after birth or cause untreatable pain and
582 distress, it is mandated that the animal, whether neonate, fetus, or embryo, must be
583 humanely euthanized before or immediately after birth. These regulations underscore
584 Australia's commitment to ensuring the welfare and ethical treatment of animal
585 embryos in research settings, aligning with broader principles of animal welfare and
586 scientific integrity [86] (updated 2021).

587
588 While regulations and laws concerning the use of embryos in research may be
589 lacking to some extent, this article aims to delve deeper into the significance of various
590 species groups in animal embryo research. By exploring the unique characteristics and
591 developmental processes of different species' embryos, we can gain a better
592 understanding of their potential as research models and the ethical considerations
593 surrounding their use. Through this discussion, we hope to shed light on the
594 importance of considering the welfare and ethical implications of embryo research
595 across different species groups.

597 5. Mammalian embryos

598
599 The development of a mammalian embryo begins with the formation of a
600 totipotent zygote during fertilization, which marks the start of embryogenesis. The
601 zygote is enclosed within the zona pellucida membrane and undergoes successive
602 cleavage divisions, leading to an increase in cell number [87]. Upon reaching the 8-cell
603 stage, compaction occurs, resulting in the formation of a compact sphere known as the
604 morula. Subsequently, cavitation occurs, which is characterized by the secretion of
605 water into the morula by the trophoblast, leading to the formation of a central
606 fluid-filled cavity called the blastocoel (Figure 1) [87].

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Figure 2. Different stages of early embryonic development in mammals.

This process involves significant structural and transcriptional modifications of the embryonic lineage as it transitions from pre- to post-implantation stages [88]. These modifications set the stage for the subsequent phase of gastrulation, which is a critical developmental stage. The blastocyst is a stage reached after four to six days of development. It is characterized by the presence of two distinct groups of cells: the inner cell mass, also known as the embryoblast, and the outer trophoblast cells [89].

The totipotent zygote has the remarkable ability to generate all embryonic tissues of the developing organism, as well as crucial extra-embryonic lineages such as the placenta and yolk sac. These additional embryonic structures are essential for the initial patterning and maintenance of fetal growth until birth [88]. A well-coordinated synchronization of cell division, morphogenesis, and differentiation is necessary to facilitate the eventual formation of the fetus [88].

Mammals exhibit considerable variation in growth and development rates during ontogeny, which strongly influences various adult phenotypes. These include allometric patterns of brain and body size and the tempo of neurodevelopment [90]. The embryonic development rate in mammals is highly variable, encompassing differences in zygote differentiation, blastulation, implantation, gastrulation, neurulation, somitogenesis, and subsequent phases of limb, facial, and brain development [90].

For more than a century, researchers have studied human development, yet the intricate molecular mechanisms driving human embryogenesis remain largely elusive, primarily due to technical challenges and ethical constraints. Consequently, mice have emerged as invaluable models for studying mammalian development extensively

[91]. Their similarities to humans in anatomy, physiology, and genetics make them ideal models for such research. Added to this is the fact that rodents are easily managed, have a fast metabolism and reproduce quickly. Moreover, advanced molecular genetic techniques for manipulating mouse and rat genomes allow for precise modifications, including gene knockouts and controlled gene expression. These methods offer valuable insights into both normal biological functions and the mechanisms underlying various diseases [91, 92].

However, despite their importance, their evolutionary distance from humans results in notable differences in biological and behavioral characteristics, potentially limiting the direct application of findings to humans [93]. For instance, certain stages of embryonic development in rodents differ significantly from those in humans, which can lead to discrepancies in research results. For example, when comparing the stages of initiation of zygote gene activation, i.e., the generation of a complete chain of transcription and gene expression, in mice and humans, there is a large temporal difference, since that occurs at the two-cell stage in mice and at the eight-cell stage in humans [88].

On the other hand, non-human primates (NHPs), due to their closer evolutionary relationship with humans, offer a more accurate model for certain biological processes. Their physiological similarities to humans enhance the validity of research findings obtained from NHP models, making them particularly valuable in biomedical research for their higher translational relevance [93].

Recent studies have examined the post-implantation development of cynomolgus monkey (*Macaca fascicularis*) embryos in a *in vitro* culture, up to and beyond gastrulation. This period is crucial for mammalian embryogenesis as it establishes connections between embryonic and maternal tissues and forms the primary germ layers and body plan. Research has shown that primate embryos go through crucial events, such as gastrulation, neurulation, and the establishment of the germ layers. These events reflect fundamental developmental milestones observed *in vivo* [94] (Table 1).

Table 1. Table of contents Mammalian embryos: List of references, country of origin, ethics committee evaluation when available, description of embryonic development stage when available and year of posting.

Nº	Name	Country	Ethics	Embrionary Stage	Year
1	<i>Dissecting primate early post-implantation development using long-term in vitro embryo culture [94]</i>	China	Not specified	6-7 days post fertilization till 20 days post fertilization	2019

2	<i>Comparison of the effects of introducing the CRISPR/Cas9 system by microinjection and electroporation into porcine embryos at different stages</i> [95]	Japan	Institutional Animal Care and Use Committee of Tokushima University (Approval Number: T28-21).	Day 0 after fertilization till 1-cell and 2-cell stage	2021
3	<i>Neurulation of the cynomolgus monkey embryo achieved from 3D blastocyst culture</i> [96].	China	Principles for the Ethical Treatment of Non-Human Primates issued by Institute of Zoology, Chinese Academy of Sciences (IOZ, CAS) Institutional Animal Care and Use Committee of the IOZ, CAS (Appl. No: IOZ-IACUC-2021-166 (for cynomolgus monkey embryo <i>in vitro</i> culture) Appl. No: IOZ-EU20191113 (for <i>in vivo</i> flushed cynomolgus monkey embryo.)) Ethics Committee of the Second Affiliated Hospital of Shandong University (Appl. No. KYLL-2021(KJ)P-0498) (for collection of human embryonic tissues)	0 day post-fertilization - 25 days post-fertilization	2023
4	<i>Cynomolgus monkey embryo model captures gastrulation and early pregnancy</i> [97]	China	Animal Advisory Committee at the CAS Center for Excellence in Brain Science and Intelligence Technology, Chinese Academy of Sciences (#CEBSIT-2020007R01)	Not specified	2023
5	<i>Tracing the emergence of primordial germ cells from bilaminar disc rabbit embryos and pluripotent stem cells</i> [98]	Japan	Unrealized (National Institute for Physiological Sciences, University of Tokyo, and Kitayama Labes Co., Ltd.)	6 to 20 days of embryonic development	2021
6	<i>Chromatin remodeling in bovine embryos indicates species-specific regulation of genome activation</i> [99]	USA	Unrealized (University of California Davis)	1-7 days post-insemination	2020
7	<i>H2AK119ub1 guides maternal inheritance and zygotic deposition of H3K27me3 in mouse embryos</i> [100]	Japan	Unrealized (Institutional Animal Care and Use Committee at RIKEN Center for Integrative Medical Sciences)	Not specified	2021

667
668 A research by Le et al. (2021) delves into optimizing gene editing techniques for
669 B4GALNT2 in pigs, a crucial step in xenotransplantation. Using the CRISPR/Cas9
670 system, the researchers examined the efficiency of gene editing via microinjection and
671 electroporation methods on porcine embryos. They found that while electroporation
672 resulted in higher blastocyst formation rates, microinjection at the 1-cell stage yielded
673 higher mutation rates compared to the 2-cell stage. These findings highlight the
674 importance of considering both the gene editing method and embryonic stage in
675 optimizing mutation efficiency [95] (Table 1).

676 In another research, scientists devised a method to study the development of
677 cynomolgus monkey embryos from the blastocyst stage to the neurula stage at d.p.f.
678 25 outside the body. This allowed them to delve into the intricate processes of
679 advanced gastrulation and early neurulation, essential for understanding embryonic
680 development. Despite facing difficulties, they managed to achieve a survival rate of
681 around 40% for cynomolgus embryos, similar to previous studies. Notably, they
682 employed a 3D culture system with specific conditions to prevent overgrowth of
683 certain cell types and promote proper embryo development, enabling longer culture
684 periods. Moreover, their analysis at the single-cell level revealed accurate DNA
685 methylation patterns in different cell types, showcasing the potential of this model for
686 epigenetic investigations. Overall, this study presents a significant advancement in
687 studying primate embryonic development outside the body [96] (Table 1).

688 689 **6. Avian embryos**

690
691 The development of a avian embryo begins with fertilization, that marks the
692 inception of development, as the oocyte expands, enveloping yolk within the plasma
693 membrane and forming the germinal vesicle. Synthesized in the maternal liver and
694 aided by follicle cells, yolk sustains the embryo's growth. Upon release, the oocyte
695 encounters sperm, penetrating the vitelline membrane to form a blastodisc. As the egg
696 journeys through the oviduct, it undergoes transformations, culminating in the
697 deposition of shell membranes and the formation of the eggshell [101]. Rapid cleavage
698 of the blastodisc ensues, with subsequent differentiation into the area opaca and area
699 pellucida, housing distinct embryonic layers. Gastrulation follows, initiated by the
700 hypoblast, forming the primitive streak and facilitating the migration of cells to form
701 endoderm and mesoderm. Neural induction and neurulation ensue, leading to the
702 establishment of a neural plate and closure of the neural tube. Later stages witness the
703 emergence of germ layers, establishment of body axes, and organogenesis, paving the
704 way for the chick's eventual hatching [101].

705 The use of avian embryos as experimental models, particularly embryos of *Gallus*
706 *gallus domesticus* (commonly referred to as rooster and chicken), has been widely
707 employed in research. Its utilization was initially described in 1911 by Rous and
708 Murphy, who sought to demonstrate the growth of chicken sarcoma tumors

709 transplanted onto the chorioallantoic membrane (CAM) of the embryo[102]. The use of
710 avian embryos has led to a reduction in study time and the use of other animals such as
711 rodents, an experimental model with embryonic development time equivalent to that
712 of the chicken embryo (CE), which are routinely used in tests. However, ethical
713 concerns regarding their use in experiments and animal welfare issues arise, including
714 challenges in estimating the quantity of generated embryos, requiring the sacrifice of
715 parent female birds, and depending on the lineage used and the scope of the research,
716 it can become expensive and time-consuming [103].

717 Chicken embryos present well-developed vascular tissues that facilitate the study
718 of complex biological systems and offer the possibility of high reproducibility in
719 studies. They are recognized as an intermediate model between *in vitro* and *in vivo*
720 research, serving as a preliminary step to mammalian studies, particularly in toxicity
721 studies and drug evaluation, where an evaluation of the organism's response is
722 necessary, which cannot be replicated *in vitro* cell culture systems [104]. It is an
723 appropriate model for evaluations, but an obstacle remains in the lack of
724 standardization of its effective use as an experimental model, particularly for drug
725 testing, where there is still no understanding of whether parameters such as
726 quantification of metabolites in the allantoic serum, counting and characterization of
727 blood cells, oxidative stress, and histological alterations are replicable variables from
728 chicken embryo to other *in vivo* experimental models [26].

729 Possessing embryonic development similar to mammals, avian embryos have
730 well-documented genetic information, whereas their physiology is less complex
731 compared to mammals, as they possess an amnion that surrounds the embryo,
732 eliminating the need for placental development and maternal influence, completing
733 their development in 21 days [103]. This experimental model also offers advantages in
734 terms of ethical and legal aspects, availability of fertilized eggs year-round at
735 affordable prices, and low maintenance costs, producing results similar to those
736 generated in murine models, thus saving time, materials, and animals used [103].

737 Within research, embryos are used for numerous parameters, including
738 angiogenesis, toxicity, ischemia, drug delivery systems, cancer development, and
739 treatment, among others [105-109] (Table 2). Emphasis is placed on studies of cancer
740 and metastasis due to the supportive environment for tumors with a large quantity of
741 blood vessels and angiogenesis [110]. They are also widely used as a host system for
742 the replication of various viruses for isolation, viral titration, and commercial vaccine
743 production due to possessing cell types that assist in viral replication [111].

744 During embryo development and its associated membranes, such as the CAM,
745 yolk sac, and amniotic sac, a diverse cellular environment is established, crucial for the
746 successful replication of various viruses. Embryonated eggs can be directly inoculated
747 onto the CAM or into the allantoic, amniotic, and vitelline sacs [112]. In coronaviruses
748 of group 3, it has been observed that inoculation of eggs through transport via the
749 allantoic or amniotic membrane provides viruses access to specific cell types that favor
750 their replication [113-115].

The infectious bronchitis virus (IBV) is capable of replicating in various epithelial surfaces of chickens, including the respiratory, gastrointestinal tract, kidney, and oviduct [116]. In the embryonated egg, IBV replicates efficiently regardless of the method of inoculation, but the allantoic route is preferred, as the virus replicates intensely in the CAM epithelium, resulting in high concentrations being shed into the allantoic fluid [117].

In comparison, turkey coronavirus (TCoV) is a virus that affects the intestinal tract, replicating only in the intestinal epithelium and the bursa of Fabricius of chickens and turkeys [115, 118, 119]. This enterotropic characteristic of TCoV is also observed during embryonic development, where the virus replicates exclusively in the embryo's intestines and the bursa of Fabricius, organs reached only through amniotic inoculation.

The embryonic phase used in research varies according to each type of experiment, whether inoculation, collection, infection, drug testing, among others, and may vary from one study to another, as the chicken embryo undergoes rapid development and a degree of maturity that changes rapidly during different phases of development [120]. It was observed that the embryonic stage used in research varies according to each type of experiment, as the degree of maturity changes rapidly during different stages of development. There was a preference among researchers for embryos in which the CAM had already formed (which begins its formation around the 3rd or 4th day of embryonic development and completely envelops the embryo and other structures by the 10th day), being used as one of the main routes of substance administration [120] (Table 2).

Table 2. Table of contents Avian embryos: List of references, country of origin, ethics committee evaluation when available, description of embryonic development stage when available and year of posting.

Nº	Name	Country	Ethics	Embrionary Stage	Year
1	<i>An In Ovo Model for Testing Insulin-mimetic Compounds</i> [121].	Áustria	Unrealized (Directive 2010/63/EU)	10 to 11 days of embryonic development	2018
2	<i>Evaluation of the effect of the compound (O-Methyl)-N-(2,6-Dihydroxybenzoyl</i>	Brazil	Ethics Committee of the Federal University of Pernambuco (UFPE). (No.	25 to 49 hours of embryonic	2017

- tyramine (Riparin III) from the plant Aniba riparia (Nees) Mez (Lauraceae) on the morphogenesis of the central nervous system in Gallus gallus embryo [122].* 23076.022496/2015-89) development
- 3 *Physiological changes in chicken embryos inoculated with drugs and viruses highlight the need for more standardization of this animal model [123].* Brazil University Research and Ethics Committee Federal Government of Uberlândia (certificate A011/20 and nº 008/21). Animal 0, 3, 7, 10 e 12 days of embryonic development 2022
- 4 *Chick embryo chorioallantoic membrane model systems to study and visualize human tumor cell metastasis [110].* USA Not specified Not specified 2008
- 5 *Chick embryo chorioallantoic membrane (CAM): an alternative predictive model in acute toxicological studies for anti-cancer drugs [120].* Malaysia Unrealized. (Institutional Animal Care and Use Committee (IACUC), an Association of New England Medical Center and Tufts, the National Institutes of Health, USA) 9 and 11 days of embryonic development 2015

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|----|--|-----------|---|--|------|
| 6 | <i>Transplantability of tissues to the embryo of foreign species: its bearing on questions of tissue specificity and tumor immunity [124].</i> | USA | Not specified | 7 to 18 days of embryonic development | 1912 |
| 7 | <i>Chick Chorioallantoic Membrane (CAM) Assay as an In Vivo Model to Study the Effect of Newly Identified Molecules on Ovarian Cancer Invasion and Metastasis [106].</i> | Australia | University of Adelaide
Animal Ethics Committee | 3 to 14 days of embryonic development | 2012 |
| 10 | <i>Toxicity studies of six types of carbon nanoparticles in a chicken-embryo model [107].</i> | Poland | Unrealized.

(Polish legal regulations regarding animal experiments (DzU 2015 poz 266)) | 5, 10, 15 and 20 days of embryonic development | 2017 |
| 11 | <i>Reproductive toxicity of fluoroquinolones in birds [108].</i> | Tchéquia | Ethical Committee of the University of Veterinary and Pharmaceutical Sciences Brno, Czech Republic (accreditation by the Ministry of Agriculture of the Czech Republic No. 28414/2009–17210; project No. IGA82011). | 0 to 19 day of embryonic development | 2019 |
| 12 | <i>Embryonic toxico-pathological effects of meglumine antimoniate using a chick embryo model [109].</i> | Turkey | Animal Ethics Committee of the Research Council of the Kerman University of | 48 hours to 18 days of embryonic development | 2018 |
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Medical Sciences, Iran.

(project number

94/974/205, Ethic number

IR.KMU.REC.1394.525)

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The research led by Hrubá et al., employing egg injection techniques and a standard precocial species model, illuminates the comprehensive detrimental impacts of fluoroquinolones on avian embryonic development, notably compromising egg incubation efficacy. The adverse effects span reduced hatchability and overall hatching success, including issues such as embryonic mortality, premature hatching, deformities in chick joints, and elevated heart rate accompanied by signs of biochemical stress. Additionally, the study unveils cardiotoxic effects of fluoroquinolones in birds, evident in heightened embryonic heart rates. Noteworthy is the premature hatching phenomenon, indicating an accelerated embryonic development due to fluoroquinolone-induced cardiotoxicity. Blood biochemistry analysis upon hatching reveals markers of prolonged stress, such as hypoproteinemia, hyperglycemia, and hypertriglyceridemia, indicative of systemic physiological disruptions in treated chicks. These findings emphasize the necessity for heightened awareness of toxicity among veterinarians, particularly concerning the differential impacts of enrofloxacin and marbofloxacin in avian species, highlighting a greater risk associated with enrofloxacin administration [108] (Table 2).

Another study examined the effects of meglumine antimoniate (MA) on avian embryos, finding significant adverse impacts, particularly during the second trimester of development. The severity of effects increased with higher doses of MA. Injecting MA into the yolk sac throughout organogenesis caused distinct developmental abnormalities. Findings included hemorrhages and changes in biochemical parameters, indicating tissue injury and stress. MA exposure also hindered early vascular development, reducing expression of vascular endothelial growth factor (VEGF) and its receptor. These results suggest MA can be harmful during pregnancy, emphasizing the need for safer alternatives. The study highlights the chick embryo model's usefulness in understanding drug-induced embryonic abnormalities and urges further research on MA's safety during pregnancy [109] (Table 2).

Regarding the ethical considerations involving the use of avian embryos, it is necessary to improve the generated regulations, considering legislation such as the EU Directive 2010/63/EU, recognized worldwide, which states that embryos up to the 14th day, the first two-thirds of development, do not require approval from the ethics committee for testing [121]. Given that directives vary according to the region, it is important for the responsible researcher to verify which regulations are valid for the region where the experiment is conducted to ensure the most reliable results possible.

7. Amphibian embryos

One of the most studied animal classes in research due to its ease of study is amphibians [125-127]. As anamniotic vertebrates, they lack an amnion surrounding the embryo; however, the development of amphibians and fish employs many of the same processes and genes used by other vertebrates to generate body axes and organs [126].

The abdomen of an adult female *Xenopus laevis* is filled with thousands of large oocytes measuring 1.2 mm in diameter. When removed from the mother, the oocytes can be cultured for several weeks in a simple saline solution [128]. Microinjected oocytes have led to many advances in gene expression analysis in vertebrates [128]. In addition to their large size, the ova of female *X. laevis* exhibit unequal distribution of various molecules, resulting in a bicolouration visible to the naked eye [129, 130], due to polarity differences, changes in the cytoplasm and yolk, nucleus position, among other characteristics [130]. When fertilized by a spermatozoon from a male *X. laevis*, the less dense pigmented part of the ovum rotates to the top and releases the embryo into the vitelline envelope [130]. While the fertilized *ovum* immediately replicates the nucleus, the oocyte remains metabolically active but unchanged under simple culture conditions for a few weeks [128].

After fertilization, a cascade of events begins where the embryo's cortex rotates relative to the deep region, which will give rise to the dorsal side of the embryo, also known as the "gray crescent" [130]. For many experiments, it is important to predict the location where the dorsoventral axis of the embryo will form, some techniques involving dyes are applied to predict this event [130]. Then, several cycles of cell division occur, transforming the zygote into numerous smaller cells with a wide variety of genes expressed in specific patterns that interact in different ways to establish other patterns of gene expression [129]. The cells of the "gray crescent," which were clustered on one side of the embryo, form the blastula phase, creating a cluster of cells with a cavity in the center [129]. After this phase, the embryo enters the gastrula phase, where cell migration into the embryo causes a folding of the tissue inward [129]; the term used to describe the location where cells migrate into the embryo is "blastopore".

However, during the early stages of developmental genetics research, amphibian embryos were of little use, partly due to the long period of growth of these animals before becoming fertile and because their chromosomes are often found in multiple copies, making the process of mutagenesis difficult [126]. Nevertheless, with the introduction of molecular techniques, such as *in situ* hybridization, chromatin immunoprecipitation, and dominant-negative proteins, researchers were able to return to studies using amphibian embryos, enabling the integration of molecular analyses with previous experimental findings [126].

From the 1880s, experimental embryologists promoted the use of amphibians using local species from Europe and North America [131]. By the 1930s to the 1960s, the frog *X.*

856 *laevis*, originating from South Africa, was introduced into European and North
857 American laboratories [131].

858 Among the advantages of using *X. laevis* as an experimental system for studying
859 early embryonic development are the ease of animal maintenance, availability of large
860 quantities of embryos throughout the year, and rapid embryo development [125, 132].
861 The embryos are easily manipulated for studies involving tissue transplantation,
862 recombination, and implant cultures [132]. Due to its relatively large embryo size, *X.*
863 *laevis* allows efficient isolation of specific regions of embryonic tissues, enabling the
864 supply of sufficient amounts of initial materials for cDNA library construction [132].

865 Currently, the understanding of embryogenesis is largely associated with the
866 control of gene expression through various signaling pathways. Many of these
867 embryonic signaling pathways that allow embryological events are related to various
868 diseases lacking effective treatments or even their cure. *X. laevis* embryos have been
869 shown to be an effective tool in the search for compounds that affect embryonic
870 signaling pathways, thanks to their high number and size of eggs, rapid embryo
871 development, and susceptibility to pharmacological, surgical, and genetic techniques
872 [125, 132]. In addition to the previous applications, the embryonic development of the
873 optic pathway of *X. laevis* is one of the most well-understood experimental models for
874 axon localization [133]. They are also considered established experimental models with
875 applications for toxicological evaluation of chemical substances and identification of
876 drugs with potential teratogenic risks [125].

877 The use of amphibian embryos involves various phases of embryonic development,
878 depending on the research objectives. However, some specific phases are more
879 commonly used, including the four-cell embryo phase [125], dorsal marginal zone [134],
880 blastula [127], and gastrulation [125]. In addition, various studies involve the
881 Wnt/ β -catenin signaling pathway responsible for embryonic development, with
882 organogenesis, cell differentiation, polarization, and migration [134] (Table 3).
883 Furthermore, the aquatic nature of embryos, coupled with their independence from
884 maternal influence, facilitates their exposure and absorption of exogenous hormones or
885 other chemical compounds. This enables the study of developmental deficits and
886 functional alterations caused by chemical substances in the embryo [135]. Studies on *X.*
887 *laevis* tissues/organs have significantly contributed to the current understanding of
888 embryonic development, spanning areas such as the brain, eyes, heart, and kidneys, due
889 to their ease of manipulation and molecular intervention [135]. Fragile X Syndrome and
890 other neurodevelopmental disorders are among the human conditions in which the use
891 of amphibian embryos allows for a better understanding through behavioral and
892 electrophysiological experiments [136]; the most commonly used stages are tadpoles in
893 the limb bud development phase [137].

894
895 **Table 3.** Table of contents Amphibian embryos: List of references, country of origin, ethics
896 committee evaluation when available, description of embryonic development stage when available
897 and year of posting

Nº	Name	Country	Ethics	Embryonic Stage	Year
1	<i>Effects of Natural Compounds on Xenopus Embryogenesis: A Potential Read Out for Functional Drug Discovery Targeting Wnt/-catenin Signaling</i> [134]	Brazil	Not specified	Two-cell stage embryos (0:45 hour post fertilization (hpf)) - stage 10.5 (11:29 hpf)	2012
2	<i>Aminomethylphosphonic acid (AMPA) alters oxidative status during embryonic development in an amphibian species</i> [138]	France	COMETHEA ethic committee and Ministère de L'Enseignement Supérieur, de la Recherche et de L'innovation) under permit #APAFIS#13477–2018032614077834 v7.	16 days of embryonic development	2022
3	<i>Glyphosate without Co-formulants affects embryonic development of the south african clawed frog Xenopus laevis</i> [139]	German y	Not specified	Fertilization (2 cell stage) - Stage 44/45 embryos (approx. 14 incubation days)	2023
4	<i>Nicosulfuron, a sulfonylurea herbicide, alters embryonic development and oxidative status of hatchlings at environmental concentrations in an amphibian species</i> [140]	France	COMETHEA ethic committee and Ministère de L'Enseignement Supérieur, de la Recherche et de L'innovation) under permit #APAFIS#13477–2018032614077834 v7.	12 days of embryonic development	2022
5	<i>Metal ion fluxes controlling amphibian fertilization</i> [141]	USA	Northwestern University Institutional Animal Care and Use Committee (NU-IACUC, animal assurance no. D16-00182), under protocol IS00008873 (LaBonne) National Institutes of Health's Guide for the Care and Use of Laboratory Animals	Not specified	2021

6	<i>Morphological and Transcriptomic Analyses Reveal the Toxicological Mechanism and Risk of Nitrate Exposure in Bufo gargarizans Embryos [142]</i>	China	Ethics Committee of Animal Ethical and Welfare Committee of Wenzhou University (WZU-039, 2021.02.28)	7-11 day after exposition to different treatments	2024
7	<i>Alpha-tocopherol exerts protective function against the mucotoxicity of particulate matter in amphibian and human goblet cells [143]</i>	South Korea	Institutional Animal Care and Use Committee (IACUC) of Ulsan National Institute of Science and Technology (UNIST) approved this work (Reference number, UNISTIACUC-16-14)	Not specified	2020
8	<i>Developmental Toxicity Assessment of a Chlorothalonil-Based Fungicide in a Native Amphibian Species [144]</i>	Argentina	Institutional committee for the care and use of animals in experimentation (CICUAE) of the National University of San Martín (UNSAM)	Early blastula stage, S.4 - 504 h (21 days)	2020

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A study demonstrated that the primary metabolite of glyphosate, AMPA, alters oxidative status during embryonic development in an amphibian species found in agricultural landscapes, even at environmentally relevant concentrations. The effects were observed in markers of oxidative status, such as thiols and the SOD/(CAT + GPx) ratio, which showed non-monotonic responses to AMPA exposure. Interestingly, while low concentrations of AMPA influenced the levels of thiols, they did not affect CAT levels, suggesting complex responses to different concentrations of the contaminant. Additionally, the study highlights the importance of antioxidants in protecting early development and suggests that AMPA exposure may induce non-monotonic effects, potentially stimulating antioxidant mechanisms or selecting embryos resistant to oxidative stress [138] (Table 3).

Another study investigated the impact of glyphosate (GLY) without co-formulants on *X. laevis* embryogenesis, using concentrations from 0.1 to 243 mg/L to simulate environmental exposures. Results revealed impaired embryonic development, with reductions in body length, head area, and eye area observed even at concentrations as low as 0.1 mg/L GLY, consistent across various concentrations tested [139]. Moreover, GLY-treated embryos exhibited increased mobility at later stages, indicating potential neurological effects. Additionally, GLY interfered with cranial nerve formation and cardiac development, leading to structural abnormalities and decreased heart rate. The investigation into GLY's impact on cardiac cell differentiation showed significant reductions in *mhα* gene expression, suggesting an early negative influence on heart cell differentiation. However, GLY treatment did not

921 affect cell proliferation or apoptosis rates during *X. laevis* embryo development at
922 stage 28. Comparison with previous studies on GLY without co-formulants and GLY
923 formulations across different organisms revealed consistent morphological, functional,
924 and molecular alterations, indicating that GLY alone drives these effects [139] (Table
925 3).

926 8. Reptile embryos

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929 The development of reptile embryos involves two distinct aspects: differentiation,
930 responsible for the origin of tissues and organic systems, and growth, which consists of
931 increasing the size of the embryo. Both differentiation and embryo growth occur in
932 parallel with the development of extraembryonic membranes [145].

933 The differentiation of embryonic phases begins with neurulation, used as a
934 reference point in embryological studies due to its frequency and clarity [145]. When the
935 mesodermal layer divides to form the extraembryonic coelom, the definitive yolk sac is
936 constituted by the vascularized mesoderm-endoderm layer internally (splanchnopleure)
937 [145]. Even though it is the only vascularized membrane in contact with the shell, the
938 embryo's oxygen demands are minimal at this point [145].

939 The allantois arises as the last extraembryonic membrane, originating as a growth
940 of the posterior intestine (endoderm and mesoderm), usually coinciding with the
941 completion of the amnion and shortly before or at the same time as the limbs form. The
942 vascularized chorioallantoic membrane internally lines the shell and plays a crucial role
943 in gas exchange through the shell, replacing the yolk sac as a respiratory surface in many
944 species. The temporal development of the CAM is not fully understood, and there is no
945 clear pattern in the available data [145].

946 In general, tissue differentiation, organogenesis, including gonads and body
947 structures, occur in the first 30-40% of the development period. At this stage, embryos
948 begin to resemble birds, lizards, turtles, etc., although they have reached less than 5% of
949 their mass at hatching. Once the embryo and its supporting structures are formed,
950 growth becomes the most prominent feature of development [145].

951 Reptile embryos exhibit movement within the egg, responding to external
952 environmental temperature [146-148], suggesting embryonic thermoregulation,
953 behaving similarly to free-living stages, with one of the embryonic behaviors directly
954 related to adaptation [149-151].

955 Turtles are more frequently used in experiments, as they contain more studies
956 related to embryonic development and movement compared to other reptile species,
957 usually producing large eggs that increase the feasibility of embryos experiencing
958 thermal gradients [152].

959 The first described report of embryonic thermotaxis was observed in the Chinese
960 softshell turtle (*Pelodiscus sinensis*), where embryos moved from the neck point within
961 the shell towards a consistently estimated external heat source, adjusting their bodies
962 within the egg by approximately 30° [148], considering the average nest temperatures in

nature generally range from 20 to 30°C and extreme temperatures vary from 10 to 45°C [145, 153].

Behavioral thermoregulation within the egg has significant impact and importance for the embryo, enabling direct control of various relevant characteristics aiding in development [148], such as performances, size, energy, and occasionally sex [154-157]. However, the rate of temperature variation within eggs reverses direction twice a day [158], requiring embryo position changes within the egg, demanding hours to track temperature preferences. With embryos taking days to change position, they lack the ability to effectively track the thermal environment [158], requiring more data to evaluate the speed at which reptile embryos can generally move in response to temperature.

After suggesting transgenesis in reptiles, Modzdiak and Petite (2010) [159] produced transgenic snakes, culminating in the recent production of the first transgenic albino lizard using CRISPR-Cas9-mediated genetic editing [160]. Transgenic reptiles such as snakes, lizards, and crocodiles are used as models in tests of oncogenic and teratogenic agents [161], to evaluate toxic, teratogenic, and/or carcinogenic substances, similar to fluorescent GloFish zebrafish or genetically modified frogs. These transgenic reptiles are particularly useful for examining the effects of these substances at different concentrations and conditions, mimicking their natural habitat, including androgens, estrogens, other endocrine disruptors, and pesticides, which are commonly found in the environment where these reptiles live [159].

Certain species such as snakes and lizards are widely used as models for genetic developmental studies. Turtle embryos, specially *Chelonia mydas*, are one of the most popular models in developmental and anatomical biology research [162]. Additionally, lizard species such as *Lacerta trilineata*, *Anolis carolinensis*, and *Eublepharis macularius* are the reptiles most commonly used in comparative research in the field of developmental biology [163]. The use of reptile embryos still lacks related research overall, due to the fact that studies in bird embryos are more commonly used regarding embryonic structures and phases [164].

Table 4. Table of contents Reptile embryos: List of references, country of origin, ethics committee evaluation when available, description of embryonic development stage when available and year of posting.

Nº Name	Country	Ethics	Embryonary Stage	Year
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1	<i>Molecular sensors for temperature detection during behavioral thermoregulation in turtle embryos [54].</i>	China	Guide for the Care and Use of Laboratory Animals of Institute of Zoology, Chinese Academy of Sciences. Animal Ethics Committee at the Institute of Zoology, Chinese Academy of Sciences (IOZ14001).	Not specified	2021
2	<i>Do Microbiota in the Soil Affect Embryonic Development and Immunocompetence in Hatchling Reptiles? [165]</i>	Australia	Parks and Wildlife Commission of the Northern Territory (approval 47830) The University of Sydney Animal Ethics Committee (approval # 2013/6010)	24 h of oviposition to 6 weeks	2022
3	<i>Influence of incubation temperature on embryo development, hatchling morphology and early growth rate in red-footed tortoise (<i>Chelonoidis carbonaria</i>) [166].</i>	Brazil	Animal Care and Use Committee (CEUA, FCAV-UNESP, Jaboticabal protocol number 006094/19).	0–4 days old till 3 months	2021
4	<i>Gene expression of the IGF hormones and IGF binding proteins across time and tissues in a model reptile [167].</i>	USA	IACUC 2017-3027	Preoviposition - 20 days postoviposition	2020
5	<i>Behavioral thermoregulation by reptile embryos promotes hatching success and synchronization [168].</i>	China	Guide for the Care and Use of Laboratory Animals of Institute of Zoology, Chinese Academy of Sciences. Animal Ethics Committee at the Institute of Zoology, Chinese Academy of Sciences (IOZ14001).	Not specified	2023
6	<i>Transcriptomic analysis of preovipositional embryonic arrest in a nonsquamate reptile (<i>Chelonia mydas</i>) [169].</i>	Australia	Animal Ethics Committee of Monash University's School of Biological Sciences (approval BSCI/2018/15). Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.	0 h - 36 h after oviposition	2022
7	<i>Increasing hypoxia progressively slows early embryonic development in an oviparous reptile, the green turtle, <i>Chelonia mydas</i> [170].</i>	Australia	Monash University's Biological Sciences Animal Ethics Committee (Approval no. 25519) Egg collection was conducted under the authority of a scientific research permit	Not specified	2022

(P-PTUKI-100044632) issued by the Queensland Department of Environment and Science.

8	<i>Moderate climate warming scenarios during embryonic and post-embryonic stages benefit a cold-climate lizard [171].</i>	China	Animal Ethics Committees at the Institute of Zoology, Chinese Academy of Sciences (IOZ14001) .	Not specified	2022
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A research conducted by Gárriz et al. (2022) sheds light on the molecular mechanisms underlying hypoxia-induced embryonic arrest in green sea turtles (*Chelonia mydas*), providing valuable insights into the transcriptional changes associated with this phenomenon. Their findings indicate a significant downregulation of genes involved in key developmental processes such as DNA replication and cell cycle progression, coupled with an upregulation of genes targeted by hypoxia-inducible factors (HIFs), which are typically suppressed during normal development. These results suggest that while hypoxia-induced arrest appears to be unique to turtles among oviparous reptiles, the underlying genetic pathways may be conserved across different species. Moreover, the study highlights the importance of understanding these mechanisms for conservation efforts aimed at protecting endangered turtle species [169] (Table 4).

In parallel, the study by Adams et al. (2022) complements these findings by demonstrating the physiological consequences of hypoxic conditions on embryonic development in green sea turtles. Their research shows that hypoxia delays the onset of embryonic development and reduces the growth rate of turtle embryos, indicating a direct relationship between oxygen concentration and developmental progression. Importantly, the study underscores the need for careful management of oxygen levels in conservation practices involving turtle eggs, as even moderate hypoxia can significantly impact embryonic growth and subsequent hatchling survival [170] (Table 4). Together, these studies provide a comprehensive understanding of the challenges posed by hypoxia to the early life stages of green sea turtles, highlighting the urgent need for further research to mitigate these impacts in the face of environmental changes.

9. Fish embryos

Fish exhibit diverse reproductive strategies influenced by environmental factors. These include hermaphroditism, parthenogenesis, and gonochorism, as well as oviparous and viviparous reproduction [172]. Additionally, there is also the existence of diapause, i.e., dormancy in embryonic development, in some fish populations due to environmental influences[173]. This reproductive plurality allows for the existence of

multiple characteristics that make the embryos of these animals promising experimental models for studies ranging from the functioning of genetic mechanisms to ecotoxicity in environments [174, 175].

The embryonic development of fish passes through stages of fertilization, cleavage (which is meroblastic discoidal) [129], blastula, gastrula, segmentation, and subsequent incubation, during which organogenesis occurs. It is noteworthy that in the early stages, the embryo's DNA is protected from damage by deposits of maternal mRNA [176].

Among the embryos of various animal species, the most prominent ones used as models in research are the zebrafish (*Danio rerio*) and the Japanese medaka (*Oryzias latipes*) [172].

In this work, we will focus on the one with the greatest prominence, the zebrafish, as its embryo is the most commonly used model [177]. The first documented use of this animal for such purposes dates back to 2002 [178], although existing research on its development dates back to 1937 [179]. It is noteworthy that the utilization of zebrafish embryos is advantageous as a model for human conditions and related genetic studies, owing to the presence of approximately 70% orthology among genomes, as well as the ease of developing gene overexpression and knockdown processes [180, 181].

In the evaluated studies, there is no standardized stage of embryonic development at which the embryos are utilized; they are mostly maintained throughout their evolution in the tests. However, they are analyzed concerning their stages to assess the ongoing validity thereof, while considering whether the morphological and physiological changes during experimental development arise from the tests conducted or from embryonic changes [59]. The treatment timing of the embryos is typically indicated in terms of hours or days post-fertilization. Descriptions of the embryonic stages used in the reference papers for constructing this text are available in Table 5.

In this context, the primary uses for this model pertain to studies involving toxicity, efficacy, and testing of drugs and substances with potential toxicity. This can be seen in the work developed by Nair et al. in 2021 [182], creating a detailed protocol on how to harness the study potential of toxic compounds in this model, indicating how to measure the maximum tolerable concentration (MTC), lethal concentration, and compound interaction throughout embryonic and larval development. Such work demonstrates the model's effectiveness while establishing a standard for its use.

Another study demonstrating this embryo as an effective model for toxic compound studies involves the detection of cadmium, a heavy metal of medical importance. Developed by Blechinger *et al.* [183], this study used embryos genetically labeled with fluorescent probes that respond to different amounts of cadmium in the environment, creating an efficient and reproducible means of indicating the presence and quantity of the metal in the medium.

Molecules with defensive potential against the latter and antioxidant power can also be detected, as shown by Arteaga *et al.* [184], their study demonstrates that zebrafish embryos are models that can replace *in vitro* antioxidant assays to represent the capacity of molecules in an *in vivo* context. In this work, nine natural antioxidants in

1071 food are used in treatment with a potent oxidant in the presence of embryos, ultimately
 1072 demonstrating the defense capability of several of these molecules.

1073 **Table 5.** Table of contents Fish embryos: List of references, country of origin, ethics committee
 1074 evaluation when available, description of embryonic development stage when available and year of
 1075 posting.

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Nº	Name	Country	Ethics	Embryonary Stage	Year
1	<i>Embryo ecology: Developmental synchrony and asynchrony in the embryonic development of wild annual fish populations [173]</i>	Czech Republic and USA	Not specified	All stages	2021
2	<i>Brazilian silverside, <i>Atherinella brasiliensis</i> (Quoy & Gaimard, 1825) embryos as a test-species for marine fish ecotoxicological tests [175]</i>	Brazil	CEUA-Universidade Federal do Rio de Janeiro (nº 063/17), Brazilian Ministry of the Environment (MMA)/Chico Mendes Institute for Biodiversity Conservation (ICMBio) (Permits nº 43874-1 and 43874-2)	Blastula stage	2021
3	<i>Developmental toxicology of cadmium in living embryos of a stable transgenic zebrafish line [183]</i>	USA	Not specified	All stages	2002
9	<i>Observations of the early development of the zebra fish, <i>Brachydanio rerio</i> [179]</i>	USA	Not specified	All stages	1937
10	<i>The zebrafish reference genome sequence and its relationship to the human genome [180]</i>	Multinational	Not specified	Not specified	2013
11	<i>The Zebrafish Embryo as a Model Organism for Testing mRNA-Based Therapeutics [181]</i>	Belgium/ Netherlands	Not specified	All stages	2023
12	<i>Stages of embryonic</i>	USA	Not specified	All stages	1995

	<i>development of the zebrafish [59]</i>				
13	<i>Systematic Evaluation of the Effects of Toxicant Exposure on Survival in Zebrafish Embryos and Larvae [182]</i>	United Arab Emirates	Not specified	Not specified	2021
14	<i>The Zebrafish Embryo as a Model to Test Protective Effects of Food Antioxidant Compounds [184]</i>	Spain	Department of Livestock and Fisheries of the Government of Catalonia – protocol 7971	0 – 48 hours post-fertilization	2021
16	<i>Quantifying endothelial cell proliferation in the zebrafish embryo [185]</i>	UK	UK Home Office - Project Licence 70/8588	< 5 days post-fertilization	2021

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The potentialities shown by the above-mentioned works are primarily due to the transparency of this embryo, allowing for the analysis of phenotypic changes caused by such substances, its diminutive size, and the ease of maintaining them in large numbers, owing to their high reproductive capacity and low cost, thus providing a high potential for assay repetition. [186].

Is further important to note that the use of zebrafish embryos as experimental models regarding ethical considerations still requires improvements and advancements, as many legislations, such as the European Union's Directive 2010/63/EU, one of the most advanced legal frameworks in animal experimentation available, upon which many countries base their laws in this context, do not recognize embryos before the stage of independent feeding, up to 5 days post-fertilization, as an animal model, and are not protected by laws pertinent to these [187, 188] . However, some local legislations have specific requirements that demand analysis when using embryos as models. Such details are available regarding the reference works in Table 5.

Therefore, it is the responsibility of the researcher to always be attentive to the legal issues of their country before experimentation, as well as to always take into account the main bioethical principles when using such embryos as models, to ensure reliable and quality research [189].

10. Conclusions

Each type of embryonic model, whether mammalian, avian, amphibian, reptilian, or piscine, offers unique advantages for scientific investigation. Mammalian embryos provide insights into complex developmental processes, while avian embryos boast a vascular-rich environment conducive to studying physiological changes. Amphibian embryos, like those of salamanders and frogs, are commonly used to explore embryonic development and physiological alterations. Despite the benefits, ethical

1105 considerations are paramount. The lack of universal legislation addressing embryonic
1106 model use highlights gaps in regulatory frameworks, necessitating a focus on ethical
1107 practices and animal welfare. By prioritizing collaborative efforts and global ethical
1108 standards, leveraging embryonic models can catalyze significant advancements in
1109 scientific understanding and medical progress. Integrating these alternative methods
1110 with traditional animal models promises more ethical and effective research practices
1111 and replacement in the future.

1112
1113 **Supplementary Materials:** The following supporting information can be downloaded at:
1114 www.mdpi.com/xxx/s1, Figure 1. Animal embryos and their most common uses in research; Table
1115 1: Table of contents Mammalian embryos: List of references, country of origin, ethics committee
1116 evaluation when available, description of embryonic development stage when available and year
1117 of posting; Figure 2. Different stages of early embryonic development in mammals; Table 2: Table
1118 of contents Avian embryos: List of references, country of origin, ethics committee evaluation when
1119 available, description of embryonic development stage when available and year of posting;
1120 Table 3: Table of contents Amphibian embryos: List of references, country of origin, ethics
1121 committee evaluation when available, description of embryonic development stage when
1122 available and year of posting; Table 4: Table of contents Reptile embryos: List of references,
1123 country of origin, ethics committee evaluation when available, description of embryonic
1124 development stage when available and year of posting; Table 5: Table of contents Fish embryos:
1125 List of references, country of origin, ethics committee evaluation when available, description of
1126 embryonic development stage when available and year of posting.

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1128 participated in the search for articles to be reviewed and carried out the writing of the review.
1129 K.P.L., designated figures. M.C.d.O.F. and M.M.N. designated tables. I.L.d.L, F.B.F. and M.V.d.S.
1130 participated in the supervision and review of this review. Funding acquisition, M.V.d.S. All
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1142 References

- 1143 1. Andersen, M.L. and L.M. Winter, *Animal models in biological and biomedical research-experimental and ethical concerns*. Anais
1144 da Academia Brasileira de Ciências, 2017. **91**: p. e20170238.
- 1145 2. Gibbs, R.A., et al., *Genome sequence of the Brown Norway rat yields insights into mammalian evolution*. Nature, 2004. **428**(6982):
1146 p. 493-521.
- 1147 3. Lunney, J.K., et al., *Importance of the pig as a human biomedical model*. Science Translational Medicine, 2021. **13**(621): p.
1148 eabd5758.
- 1149 4. Hart, E.A., et al., *Lessons learned from the initial sequencing of the pig genome: comparative analysis of an 8 Mb region of pig*
1150 *chromosome 17*. Genome Biol, 2007. **8**(8): p. R168.
- 1151 5. Ablain, J. and L.I. Zon, *Of fish and men: using zebrafish to fight human diseases*. Trends Cell Biol, 2013. **23**(12): p. 584-6.
- 1152 6. Mukherjee, P., et al., *Role of animal models in biomedical research: a review*. Laboratory Animal Research, 2022. **38**(1): p. 18.

- 1153 7. Vashishat, A., et al., *Alternatives of Animal Models for Biomedical Research: a Comprehensive Review of Modern Approaches*. Stem
1154 Cell Reviews and Reports, 2024: p. 1-19.
- 1155 8. Andersen, M.L. and L.M.F. Winter, *Animal models in biological and biomedical research - experimental and ethical concerns*.
1156 Anais da Academia Brasileira de Ciencias, 2019. **91**(suppl 1): p. e20170238.
- 1157 9. Clift, M.J. and S.H. Doak, *Advanced in vitro models for replacement of animal experiments*. 2021, Wiley Online Library. p.
1158 2101474.
- 1159 10. Herrmann, K., K. Jayne, and C. Redmond, *Animal Experimentation: Working Towards a Paradigm Change*. Chapter 27 When
1160 Is an Alternative Not an Alternative? Supporting Progress for Absolute Replacement of Animals in Science. 2019: Brill.
1161 654-672.
- 1162 11. Mushtaq, S., Y.K. Das, and A. Aksoy, *Alternative Methods to Animal Experiments*. Turkiye Klinikleri Journal of Medical
1163 Sciences, 2018. **38**: p. 161-170.
- 1164 12. Nagarajan, P., R. Gudde, and R. Srinivasan, *Essentials of Laboratory Animal Science: Principles and Practices*. 2021: Springer
1165 Singapore, Imprint: Springer.
- 1166 13. Zosen, D., et al., *Chicken embryo as animal model to study drug distribution to the developing brain*. Journal of Pharmacological
1167 and Toxicological Methods, 2021. **112**: p. 107105.
- 1168 14. Takahi, M., et al., *Xenograft of human pluripotent stem cell-derived cardiac lineage cells on zebrafish embryo heart*. Biochemical
1169 and Biophysical Research Communications, 2023. **674**: p. 190-198.
- 1170 15. Juárez-Portilla, C., et al., *El uso de los animales en la investigación y en la enseñanza: lineamientos y directrices para su manejo*.
1171 Revista Eduscientia. Divulgación de la ciencia educativa, 2019. **2**(4): p. 4-19.
- 1172 16. Neves, L.M., T.A. Wilgus, and A. Bayat, *In vitro, ex vivo, and in vivo approaches for investigation of skin scarring: Human and
1173 animal models*. Advances in Wound Care, 2023. **12**(2): p. 97-116.
- 1174 17. Hickman, D., et al., *Commonly Used Animal Models*. 2017. p. 117-175.
- 1175 18. Morales, M.M., *Métodos alternativos à utilização de animais em pesquisa científica: mito ou realidade?* Ciência e Cultura, 2008. **60**:
1176 p. 33-36.
- 1177 19. Langhans, S.A., *Using 3D in vitro cell culture models in anti-cancer drug discovery*. Expert Opinion on Drug Discovery, 2021.
1178 **16**(8): p. 841-850.
- 1179 20. Roth, A. and M.-W. Berlin, *Human microphysiological systems for drug development*. Science, 2021. **373**(6561): p. 1304-1306.
- 1180 21. Cardoso, B.D., et al., *Recent advances on cell culture platforms for in vitro drug screening and cell therapies: From conventional to
1181 microfluidic strategies*. Advanced Healthcare Materials, 2023. **12**(18): p. 2202936.
- 1182 22. Zink, D., J.K.C. Chuah, and J.Y. Ying, *Assessing toxicity with human cell-based in vitro methods*. Trends in molecular medicine,
1183 2020. **26**(6): p. 570-582.
- 1184 23. Blay, V., et al., *High-throughput screening: today's biochemical and cell-based approaches*. Drug Discovery Today, 2020. **25**(10): p.
1185 1807-1821.
- 1186 24. Kapila, R., S. Kapila, and R. Vij, *Efficacy of Milk-Derived Bioactive Peptides on Health by Cellular and Animal Models*. 2017. p.
1187 303-311.
- 1188 25. Stampfl, A., et al., *Langendorff heart: a model system to study cardiovascular effects of engineered nanoparticles*. ACS Nano, 2011.
1189 **5**(7): p. 5345-53.
- 1190 26. Jota Baptista, C.V., A.I. Faustino-Rocha, and P.A. Oliveira, *Animal models in pharmacology: A brief history awarding the nobel
1191 prizes for physiology or medicine*. Pharmacology, 2021. **106**(7-8): p. 356-368.
- 1192 27. Commission, E., *2019 report on the statistics on the use of animals for scientific purposes in the Member States of the European
1193 Union in 2015-2017*, in REPORT FROM THE COMMISSION TO THE EUROPEAN PARLIAMENT AND THE COUNCIL.
1194 2020.

- 1195 28. Care, C.C.o.A., *CCAC animal data report 2019*. 2020.
- 1196 29. Carbone, L., *Estimating mouse and rat use in American laboratories by extrapolation from Animal Welfare Act-regulated species*.
1197 Scientific Reports, 2021. **11**(1): p. 493.
- 1198 30. Doyle, A., et al., *The construction of transgenic and gene knockout/knockin mouse models of human disease*. Transgenic Res, 2012.
1199 **21**(2): p. 327-49.
- 1200 31. Waterston, R.H., et al., *Initial sequencing and comparative analysis of the mouse genome*. Nature, 2002. **420**(6915): p. 520-62.
- 1201 32. Fan, J., et al., *Principles and applications of rabbit models for atherosclerosis research*. Journal of atherosclerosis and thrombosis,
1202 2018. **25**(3): p. 213-220.
- 1203 33. Benchoula, K., et al., *The promise of zebrafish as a model of metabolic syndrome*. Exp Anim, 2019. **68**(4): p. 407-416.
- 1204 34. Burggren, W.W. and S. Warburton, *Amphibians as Animal Models for Laboratory Research in Physiology*. ILAR Journal, 2007.
1205 **48**(3): p. 260-269.
- 1206 35. Doke, S.K. and S.C. Dhawale, *Alternatives to animal testing: A review*. Saudi Pharmaceutical Journal, 2015. **23**(3): p. 223-229.
- 1207 36. Langford, D.J., et al., *Coding of facial expressions of pain in the laboratory mouse*. Nature methods, 2010. **7**(6): p. 447-449.
- 1208 37. Van Norman, G.A., *Limitations of Animal Studies for Predicting Toxicity in Clinical Trials: Is it Time to Rethink Our Current
1209 Approach?* JACC: Basic to Translational Science, 2019. **4**(7): p. 845-854.
- 1210 38. Husain, A., et al., *A Review on Alternative Methods to Experimental Animals in Biological Testing: Recent Advancement and
1211 Current Strategies*. J Pharm Bioallied Sci, 2023. **15**(4): p. 165-171.
- 1212 39. Verdi, C.M., et al., *Embryonated chicken eggs: An experimental model for Pythium insidiosum infection*. Mycoses, 2018. **61**(2): p.
1213 104-110.
- 1214 40. Fernandes, M.R. and A.R. Pedroso, *Animal experimentation: A look into ethics, welfare and alternative methods*. Rev Assoc Med
1215 Bras (1992), 2017. **63**(11): p. 923-928.
- 1216 41. Dhillon, S.S., et al., *Metabolic profiling of zebrafish embryo development from blastula period to early larval stages*. PLoS One, 2019.
1217 **14**(5): p. e0213661.
- 1218 42. Shiomi, M., *The History of the WHHL Rabbit, an Animal Model of Familial Hypercholesterolemia (II) - Contribution to the
1219 Development and Validation of the Therapeutics for Hypercholesterolemia and Atherosclerosis*. J Atheroscler Thromb, 2020. **27**(2):
1220 p. 119-131.
- 1221 43. Song, J., et al., *Production of immunodeficient rabbits by multiplex embryo transfer and multiplex gene targeting*. Scientific Reports,
1222 2017. **7**(1): p. 12202.
- 1223 44. Jans, V., et al., *Of mice and human embryos: is there an ethically preferred order of preclinical research on new assisted reproductive
1224 technologies?* Human Reproduction, 2018. **33**(9): p. 1581-1585.
- 1225 45. Smith, G.D., *Utility of Animal Models for Human Embryo Culture Development: Rodents*, in *Embryo Culture: Methods and
1226 Protocols*, G.D. Smith, J.E. Swain, and T.B. Pool, Editors. 2012, Humana Press: Totowa, NJ. p. 19-26.
- 1227 46. Okada, Y. and N. Hirokawa, *Chapter 14 - Observation of Nodal Cilia Movement and Measurement of Nodal Flow*, in *Methods in
1228 Cell Biology*, S.M. King and G.J. Pazour, Editors. 2009, Academic Press. p. 265-285.
- 1229 47. Speksnijder, G. and R. Ivarie, *A modified method of shell windowing for producing somatic or germline chimeras in fertilized
1230 chicken eggs*. Poult Sci, 2000. **79**(10): p. 1430-3.
- 1231 48. Spratt, N.T., Jr., *Development in vitro of the early chick blastoderm explanted on yolk and albumen extract saline-agar substrata*. J
1232 Exp Zool, 1947. **106**(3): p. 345-65.
- 1233 49. Silver, P.H.S., *Special Problems of Experimenting in ovo on the Early Chick Embryo, and a Solution*. Development, 1960. **8**(4): p.
1234 369-375.
- 1235 50. Lehel, J., et al., *Reproductive toxicological changes in avian embryos due to a pesticide and an environmental contaminant*. Acta
1236 Veterinaria Hungarica, 2021. **69**(4): p. 363-371.

- 1237 51. Roth, S., et al., *Comparative toxicity and cell-tissue distribution study on nanoparticulate iron complexes using avian embryos and*
1238 *HepG2-cells*. *Translational Research*, 2008. **151**(1): p. 36-44.
- 1239 52. Singh, S.K., D. Das, and T. Rhen, *Embryonic temperature programs phenotype in reptiles*. *Frontiers in Physiology*, 2020. **11**: p.
1240 505948.
- 1241 53. Shine, R. and W.G. Du, *How frequent and important is behavioral thermoregulation by embryonic reptiles?* *Journal of*
1242 *Experimental Zoology Part A: Ecological and Integrative Physiology*, 2018. **329**(4-5): p. 215-221.
- 1243 54. Ye, Y.-Z., et al., *Molecular sensors for temperature detection during behavioral thermoregulation in turtle embryos*. *Current Biology*,
1244 2021. **31**(14): p. 2995-3003. e4.
- 1245 55. O'Rourke, D.P., *Amphibians Used in Research and Teaching*. *ILAR Journal*, 2007. **48**(3): p. 183-187.
- 1246 56. Viriato, C., et al., *Evaluation of the potential teratogenic and toxic effect of the herbicide 2,4-D (DMA® 806) in bullfrog embryos and*
1247 *tadpoles (Lithobates catesbeianus)*. *Chemosphere*, 2021. **266**: p. 129018.
- 1248 57. Khan, P.A. and M.J. Crawford, *Regeneration and development. An amphibian call to arms*. *Developmental Dynamics*, 2021.
1249 **250**(6): p. 896-901.
- 1250 58. Wittbrodt, J., A. Shima, and M. Scharl, *Medaka—a model organism from the far East*. *Nat Rev Genet*, 2002. **3**(1): p. 53-64.
- 1251 59. Kimmel, C.B., et al., *Stages of embryonic development of the zebrafish*. *Dev Dyn*, 1995. **203**(3): p. 253-310.
- 1252 60. Iwamatsu, T., *Stages of normal development in the medaka *Oryzias latipes**. *Mech Dev*, 2004. **121**(7-8): p. 605-18.
- 1253 61. OECD, *Test No. 233: Sediment-Water Chironomid Life-Cycle Toxicity Test Using Spiked Water or Spiked Sediment*. Paris : OECD
1254 Publishing ed.
- 1255 OECD Guidelines for the Testing of Chemicals, Section 2. 2010: OECD Publishing.
- 1256 62. Lieschke, G.J. and P.D. Currie, *Animal models of human disease: zebrafish swim into view*. *Nat Rev Genet*, 2007. **8**(5): p. 353-67.
- 1257 63. Sun, M., et al., *Cardiovascular toxicity assessment of polyethylene nanoplastics on developing zebrafish embryos*. *Chemosphere*,
1258 2021. **282**: p. 131124.
- 1259 64. Vasbinder, M.A. and P. Locke, *Introduction: Global Laws, Regulations, and Standards for Animals in Research*. *Ilar j*, 2016. **57**(3):
1260 p. 261-265.
- 1261 65. Andersen, M.L. and L.M.F. Winter, *Animal models in biological and biomedical research - experimental and ethical concerns*.
1262 *Anais da Academia Brasileira de Ciências*, 2019. **91**.
- 1263 66. Committee, N.A.E.A., *Good Practice Guide*
1264 *for the use of animals in research,*
1265 *testing and teaching*. 2019.
- 1266 67. Council, N.R., et al., *Guide for the Care and Use of Laboratory Animals: Eighth Edition*. 2010: National Academies Press.
- 1267 68. Verderio, P., et al., *3Rs Principle and Legislative Decrees to Achieve High Standard of Animal Research*. *Animals*, 2023. **13**(2): p.
1268 277.
- 1269 69. Fröhlich, E. and G.D. Loizou, *Editorial: 3Rs—Strategies for reduction and refinement of animal studies*. *Frontiers in*
1270 *Pharmacology*, 2023. **14**.
- 1271 70. Union, E., *European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS*
1272 *No. 123)*. 1986: <https://www.coe.int/en/web/conventions/full-list?module=treaty-detail&treaty-num=123>.
- 1273 71. Marinou, K.A. and I.A. Dontas, *European Union Legislation for the Welfare of Animals Used for Scientific Purposes: Areas*
1274 *Identified for Further Discussion*. *Animals (Basel)*, 2023. **13**(14).
- 1275 72. Commission, E., *Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of*
1276 *animals used for scientific purposes*. *Off. J. Eur. Union*, 2010. **50**: p. 33-79.
- 1277 73. Cardon, A.D., M.R. Bailey, and B.T. Bennett, *The Animal Welfare Act: from enactment to enforcement*. *J Am Assoc Lab Anim*
1278 *Sci*, 2012. **51**(3): p. 301-5.

- 1279 74. States, U., *H.R.2409 - Health Research Extension Act of 1985*, H.-E.a. Commerce, Editor. 1985.
- 1280 75. Science, A.A.f.L. *IACUC Central*. 2019 [cited 2024 April, 04]; Available from: <https://www.aalas.org/iacuc>.
- 1281 76. Albus, U., *Guide for the Care and Use of Laboratory Animals (8th edn)*. Laboratory Animals, 2012. **46**(3): p. 267-268.
- 1282 77. Care, C.C.o.A., *CCAC guidelines: animal welfare assessment*. 2021, Canadian Council on Animal Care Ottawa, ON.
- 1283 78. Council, N.R., *The development of science-based guidelines for laboratory animal care: proceedings of the November 2003 international workshop*. 2004.
- 1284
- 1285 79. Coetser, Y.M., *An African ethical perspective on South Africa's regulatory frameworks governing animals in research*. Stud Hist
1286 Philos Sci, 2022. **92**: p. 119-128.
- 1287 80. Tanzania, U.R.o., *nimal Welfare Act, 2008 (No. 19 of 2008)*. 2008: FAO, FAOLEX.
- 1288 81. Kenya. *Act Title: PREVENTION OF CRUELTY TO ANIMALS*. 1962 [cited 2024 24/03/2024].
- 1289 82. MUÑOZ, L.I.O., *NORMA Oficial Mexicana NOM-062-ZOO-1999, Especificaciones técnicas para la producción, cuidado y uso*
1290 *de los animales de laboratorio*. 2001: https://www.gob.mx/cms/uploads/attachment/file/203498/NOM-062-ZOO-1999_220801.pdf.
- 1291 83. Brazil, *Lei Arouca*. 2008, Planalto - Brazil: https://www.planalto.gov.br/ccivil_03/ato2007-2010/2008/lei/111794.htm.
- 1292 84. Ministério da Ciência, T.e.I., *Guia brasileiro de produção, manutenção ou utilização de animais em atividades de ensino ou pesquisa*
1293 *científica / Conselho Nacional de Controle de Experimentação Animal*. 2023. p. 1107.
- 1294 85. Government, U.K. *Guidance on the operation of the Animals (Scientific Procedures) Act 1986 (ASP)*. 2017 20 December 2023
1295 [cited 2024 April, 04]; Available from:
1296 <https://www.gov.uk/guidance/guidance-on-the-operation-of-the-animals-scientific-procedures-act-1986>.
- 1297 86. Council, N., *Australian code of practice for the care and use of animals for scientific purposes*. Australian
1298 Government.[Abstract][Google Scholar], 2013.
- 1299 87. Wang, Z.C., J.Q. Zhang, and L. Li, *In vitro investigation of mammalian early embryonic development*. Yi Chuan, 2022. **44**(4): p.
1300 269-274.
- 1301 88. Molè, M., A. Weberling, and M. Zernicka-Goetz, *Comparative analysis of human and mouse development: From zygote to*
1302 *pre-gastrulation*. 2019.
- 1303 89. Mahajan, T., S. Ganguly, and N. Pagrut, *Embryogenesis: a comprehensive review*. JOURNAL OF ENTOMOLOGY AND
1304 ZOOLOGY STUDIES, 2018. **6**: p. 1151-1153.
- 1305 90. Halley, A.C., *The Tempo of Mammalian Embryogenesis: Variation in the Pace of Brain and Body Development*. Brain Behavior and
1306 Evolution, 2022. **97**(1-2): p. 96-107.
- 1307 91. Nakamura, T., et al., *Non-human primates as a model for human development*. Stem cell reports, 2021. **16**(5): p. 1093-1103.
- 1308 92. Bryda, E.C., *The Mighty Mouse: the impact of rodents on advances in biomedical research*. Mo Med, 2013. **110**(3): p. 207-11.
- 1309 93. Phillips, K.A., et al., *Why primate models matter*. Am J Primatol, 2014. **76**(9): p. 801-27.
- 1310 94. Niu, Y., et al., *Dissecting primate early post-implantation development using long-term in vitro embryo culture*. Science, 2019.
1311 **366**(6467).
- 1312 95. Le, Q.A., et al., *Comparison of the effects of introducing the CRISPR/Cas9 system by microinjection and electroporation into porcine*
1313 *embryos at different stages*. BMC Research Notes, 2021. **14**: p. 1-7.
- 1314 96. Zhai, J., et al., *Neurulation of the cynomolgus monkey embryo achieved from 3D blastocyst culture*. Cell, 2023. **186**(10): p.
1315 2078-2091. e18.
- 1316 97. Li, J., et al., *Cynomolgus monkey embryo model captures gastrulation and early pregnancy*. Cell Stem Cell, 2023. **30**(4): p. 362-377.
1317 e7.
- 1318 98. Kobayashi, T., et al., *Tracing the emergence of primordial germ cells from bilaminar disc rabbit embryos and pluripotent stem cells*.
1319 Cell reports, 2021. **37**(2).

- 1320 99. Halstead, M.M., et al., *Chromatin remodeling in bovine embryos indicates species-specific regulation of genome activation*. Nature
1321 Communications, 2020. **11**(1): p. 4654.
- 1322 100. Mei, H., et al., *H2AK119ub1 guides maternal inheritance and zygotic deposition of H3K27me3 in mouse embryos*. Nature Genetics,
1323 2021. **53**(4): p. 539-550.
- 1324 101. Mason, I., *The avian embryo: an overview*. Molecular embryology: Methods and protocols, 2009: p. 223-230.
- 1325 102. Murphy, J.B. and P. Rous, *THE BEHAVIOR OF CHICKEN SARCOMA IMPLANTED IN THE DEVELOPING EMBRYO*. J
1326 Exp Med, 1912. **15**(2): p. 119-32.
- 1327 103. Vargas, A., et al., *The chick embryo and its chorioallantoic membrane (CAM) for the in vivo evaluation of drug delivery systems*.
1328 Adv Drug Deliv Rev, 2007. **59**(11): p. 1162-76.
- 1329 104. Kue, C.S., et al., *Chick embryo chorioallantoic membrane (CAM): an alternative predictive model in acute toxicological studies for*
1330 *anti-cancer drugs*. Exp Anim, 2015. **64**(2): p. 129-38.
- 1331 105. Sommerfeld, S., et al., *Physiological Changes in Chicken Embryos Inoculated with Drugs and Viruses Highlight the Need for More*
1332 *Standardization of this Animal Model*. Animals (Basel), 2022. **12**(9).
- 1333 106. Lokman, N.A., et al., *Chick chorioallantoic membrane (CAM) assay as an in vivo model to study the effect of newly identified*
1334 *molecules on ovarian cancer invasion and metastasis*. International journal of molecular sciences, 2012. **13**(8): p. 9959-9970.
- 1335 107. Kurantowicz, N., et al., *Toxicity studies of six types of carbon nanoparticles in a chicken-embryo model*. Int J Nanomedicine, 2017.
1336 **12**: p. 2887-2898.
- 1337 108. Hruba, H., et al., *Reproductive toxicity of fluoroquinolones in birds*. BMC Veterinary Research, 2019. **15**(1): p. 209.
- 1338 109. Khosravi, A., et al., *Embryonic toxico-pathological effects of meglumine antimoniate using a chick embryo model*. PLoS One, 2018.
1339 **13**(5): p. e0196424.
- 1340 110. Deryugina, E.I. and J.P. Quigley, *Chick embryo chorioallantoic membrane model systems to study and visualize human tumor cell*
1341 *metastasis*. Histochem Cell Biol, 2008. **130**(6): p. 1119-30.
- 1342 111. Guy, J.S., *Isolation and propagation of coronaviruses in embryonated eggs*. Methods Mol Biol, 2008. **454**: p. 109-17.
- 1343 112. Senne, D., *Virus propagation in embryonating eggs*. A laboratory manual for the isolation and identification of avian
1344 pathogens, 1998: p. 235-240.
- 1345 113. Cavanagh, D. and S. Naqi, *Infectious bronchitis*. Diseases of poultry, 2003. **11**: p. 101-119.
- 1346 114. Gough, R., et al., *Isolation and identification of infectious bronchitis virus from pheasants*. 1996.
- 1347 115. Guy, J.S., *Turkey coronavirus enteritis*. Diseases of poultry, 2003. **12**: p. 330-338.
- 1348 116. Cavanagh, D., *Severe acute respiratory syndrome vaccine development: experiences of vaccination against avian infectious bronchitis*
1349 *coronavirus*. Avian pathology, 2003. **32**(6): p. 567-582.
- 1350 117. Jordan, F. and T. Nassar, *The combined influence of age of embryo and temperature and duration of incubation on the replication*
1351 *and yield of avian infectious bronchitis (IB) virus in the developing chick embryo*. Avian Pathology, 1973. **2**(4): p. 279-294.
- 1352 118. Adams, N. and M. Hofstad, *Isolation of transmissible enteritis agent of turkeys in avian embryos*. Avian diseases, 1971: p.
1353 426-433.
- 1354 119. Guy, J.S., *Turkey coronavirus is more closely related to avian infectious bronchitis virus than to mammalian coronaviruses: a review*.
1355 Avian Pathology, 2000. **29**(3): p. 207-212.
- 1356 120. Oliveira, C.E.A.d., *Ensaio da membrana corioalantoide (cam): modelo experimental como alternativa à utilização de animais em*
1357 *pesquisa e suas aplicações*, in Centro de Ciências. 2020, Universidade Federal do Ceará.
- 1358 121. Haselgrübler, R., et al., *An In Ovo Model for Testing Insulin-mimetic Compounds*. J Vis Exp, 2018(134).
- 1359 122. LIMA, L.S.d., *Avaliação do efeito do composto (O-Metil)-N-(2, 6-Diidroxibenzoil)-tiramina (Riparina III) da planta Aniba riparia*
1360 *(Nees) Mez (Lauraceae) sobre a morfogênese do sistema nervoso central em embrião de Gallus gallus*. 2017, Universidade Federal
1361 de Pernambuco.

- 1362 123. Sommerfeld, S., et al., *Physiological changes in chicken embryos inoculated with drugs and viruses highlight the need for more*
1363 *standardization of this animal model*. *Animals*, 2022. **12**(9): p. 1156.
- 1364 124. Murphy, J.B., *TRANSPLANTABILITY OF TISSUES TO THE EMBRYO OF FOREIGN SPECIES : ITS BEARING ON*
1365 *QUESTIONS OF TISSUE SPECIFICITY AND TUMOR IMMUNITY*. *J Exp Med*, 1913. **17**(4): p. 482-93.
- 1366 125. Maia, L.A., I. Velloso, and J.G. Abreu, *Advances in the use of Xenopus for successful drug screening*. *Expert Opin Drug Discov*,
1367 2017. **12**(11): p. 1153-1159.
- 1368 126. Gilbert, S.F. and M.J.F. Barresi, *Biologia do Desenvolvimento*. 2019: Artmed.
- 1369 127. Bastos, V., *Desenvolvimento e estabelecimento de linha celular de blástula de Xenopus laevis*, ed. H. Pires, H. Oliveira, and I.
1370 Lopes. 2020.
- 1371 128. De Robertis, E.M. and J.B. Gurdon, *A Brief History of Xenopus in Biology*. *Cold Spring Harb Protoc*, 2021. **2021**(12).
- 1372 129. Mauch, T.J. and G.C. Schoenwolf, *Developmental Biology. Sixth Edition. By Scott F. Gilbert*. *American Journal of Medical*
1373 *Genetics*, 2001. **99**(2): p. 170-171.
- 1374 130. Keller, R., *Early embryonic development of Xenopus laevis*. *Methods Cell Biol*, 1991. **36**: p. 61-113.
- 1375 131. Gurdon, J.B. and N. Hopwood, *The introduction of Xenopus laevis into developmental biology: of empire, pregnancy testing and*
1376 *ribosomal genes*. *The International journal of developmental biology*, 2000. **44**(1): p. 43-50.
- 1377 132. Chan, A.P. and L.D. Etkin, *Patterning and lineage specification in the amphibian embryo*. 2001.
- 1378 133. Koser, D.E., et al., *Mechanosensing is critical for axon growth in the developing brain*. *Nature neuroscience*, 2016. **19**(12): p.
1379 1592-1598.
- 1380 134. G Amado, N., et al., *Effects of natural compounds on Xenopus embryogenesis: a potential read out for functional drug discovery*
1381 *targeting Wnt/ β -catenin signaling*. *Current topics in medicinal chemistry*, 2012. **12**(19): p. 2103-2113.
- 1382 135. Gao, J. and W. Shen, *Xenopus in revealing developmental toxicity and modeling human diseases*. *Environmental Pollution*, 2021.
1383 **268**: p. 115809.
- 1384 136. Truszkowski, T.L., et al., *Fragile X mental retardation protein knockdown in the developing Xenopus tadpole optic tectum results in*
1385 *enhanced feedforward inhibition and behavioral deficits*. *Neural Development*, 2016. **11**: p. 1-12.
- 1386 137. Zahn, N., et al., *Normal Table of Xenopus development: a new graphical resource*. *Development*, 2022. **149**(14): p. dev200356.
- 1387 138. Cheron, M., et al., *Aminomethylphosphonic acid (AMPA) alters oxidative status during embryonic development in an amphibian*
1388 *species*. *Chemosphere*, 2022. **287**: p. 131882.
- 1389 139. Flach, H., et al., *Glyphosate without Co-formulants affects embryonic development of the south african clawed frog Xenopus laevis*.
1390 *Ecotoxicol Environ Saf*, 2023. **260**: p. 115080.
- 1391 140. Cheron, M., D. Costantini, and F. Brischoux, *Nicosulfuron, a sulfonylurea herbicide, alters embryonic development and oxidative*
1392 *status of hatchlings at environmental concentrations in an amphibian species*. *Ecotoxicology and Environmental Safety*, 2022. **232**:
1393 p. 113277.
- 1394 141. Seeler, J.F., et al., *Metal ion fluxes controlling amphibian fertilization*. *Nature Chemistry*, 2021. **13**(7): p. 683-691.
- 1395 142. Xie, L., et al., *Morphological and Transcriptomic Analyses Reveal the Toxicological Mechanism and Risk of Nitrate Exposure in Bufo*
1396 *gargarizans Embryos*. *Animals*, 2024. **14**(6): p. 961.
- 1397 143. Yang, H.-S., et al., *Alpha-tocopherol exerts protective function against the mucotoxicity of particulate matter in amphibian and*
1398 *human goblet cells*. *Scientific Reports*, 2020. **10**(1): p. 6224.
- 1399 144. Acquaroni, M., G. Svartz, and C. Pérez Coll, *Developmental Toxicity Assessment of a Chlorothalonil-Based Fungicide in a Native*
1400 *Amphibian Species*. *Archives of Environmental Contamination and Toxicology*, 2021. **80**(4): p. 680-690.
- 1401 145. Deeming, D., *Reptilian Incubation: Behaviour and Environment*. 2004.
- 1402 146. Zhao, B., et al., *Turtle embryos move to optimal thermal environments within the egg*. *Biology letters*, 2013. **9**: p. 20130337.

- 1403 147. Li, T., et al., *Thermoregulatory Behavior Is Widespread in the Embryos of Reptiles and Birds*. The American Naturalist, 2014.
1404 183(3): p. 445-451.
- 1405 148. Du, W.G., et al., *Behavioral thermoregulation by turtle embryos*. Proc Natl Acad Sci U S A, 2011. **108**(23): p. 9513-5.
- 1406 149. Warkentin, K.M., M.S. Caldwell, and J.G. McDaniel, *Temporal pattern cues in vibrational risk assessment by embryos of the red-eyed treefrog, *Agalychnis callidryas**. Journal of Experimental Biology, 2006. **209**(8): p. 1376-1384.
- 1407
- 1408 150. Doody, J., *Environmentally Cued Hatching in Reptiles*. Integrative and comparative biology, 2011. **51**: p. 49-61.
- 1409 151. Chapman, D.D., et al., *The behavioural and genetic mating system of the sand tiger shark, *Carcharias taurus*, an intrauterine cannibal*. Biol Lett, 2013. **9**(3): p. 20130003.
- 1410
- 1411 152. Cordero, G.A., R.S. Telemeco, and E.J. Gangloff, *Reptile embryos are not capable of behavioral thermoregulation in the egg*. Evol
1412 Dev, 2018. **20**(1): p. 40-47.
- 1413 153. Thompson, M.B., *Nest Temperatures in the Pleurodiran Turtle, *Emydura macquarii**. Copeia, 1988. **1988**(4): p. 996-1000.
- 1414 154. Janzen, F.J. and G.L. Paukstis, *Environmental sex determination in reptiles: ecology, evolution, and experimental design*. Q Rev
1415 Biol, 1991. **66**(2): p. 149-79.
- 1416 155. Jackson, K., *Herpetology: An Introductory Biology of Amphibians and Reptiles*. Phyllomedusa: Journal of Herpetology, 2014. **12**:
1417 p. 147.
- 1418 156. Birchard, G., *Effects of incubation temperature*. Reptilian Incubation-Environment, Evolution and Behavior, 2004: p. 103-123.
- 1419 157. Andrews, R. and L. Schwarzkopf, *Andrews RM, Schwarzkopf L. Thermal performance of squamate embryos with respect to climate, adult life history, and phylogeny*. Biological Journal of the Linnean Society. Biological Journal of the Linnean Society, 2012. **106**.
- 1420
- 1421 158. Telemeco, R.S., et al., *Reptile Embryos Lack the Opportunity to Thermoregulate by Moving within the Egg*. Am Nat, 2016. **188**(1):
1422 p. E13-27.
- 1423 159. Mozdziak, P.E. and J.N. Petite, *Transgenic snakes and methods of making*. 2010, Google Patents.
- 1424 160. Rasys, A.M., et al., *CRISPR-Cas9 Gene Editing in Lizards through Microinjection of Unfertilized Oocytes*. Cell Rep, 2019. **28**(9): p.
1425 2288-2292.e3.
- 1426 161. Paul E. Mozdziak, J.N.P., *TRANSGENIC REPTILES*, in
1427 <https://patentimages.storage.googleapis.com/ee/c1/cf/27721c40fa0dfe/US8134044.pdf>. 2012, North Carolina State University,
1428 Raleigh, NC (US) United States.
- 1429 162. Tokita, M. and S. Kuratani, *Normal Embryonic Stages of the Chinese Softshelled Turtle *Pelodiscus sinensis* (Trionychidae)*. Zoological Science, 2001. **18**(5): p. 705-715, 11.
- 1430
- 1431 163. Nomura, T., et al., *Genetic manipulation of reptilian embryos: toward an understanding of cortical development and evolution*.
1432 Frontiers in Neuroscience, 2015. **9**.
- 1433 164. Golkar-Narenji, A., et al., *In vitro culture of reptile PGCS to preserve endangered species*. Cell Biology International, 2023. **47**(8):
1434 p. 1314-1326.
- 1435 165. Brown, G.P. and R. Shine, *Do Microbiota in the Soil Affect Embryonic Development and Immunocompetence in Hatchling Reptiles?*
1436 Frontiers in Ecology and Evolution, 2022. **9**: p. 780456.
- 1437 166. Mendoza, P., et al., *Influence of incubation temperature on embryo development, hatchling morphology and early growth rate in red-footed tortoise (*Chelonoidis carbonaria*)*. Comparative Biochemistry and Physiology Part A: Molecular & Integrative
1438 Physiology, 2021. **259**: p. 110999.
- 1439
- 1440 167. Beatty, A.E. and T.S. Schwartz, *Gene expression of the IGF hormones and IGF binding proteins across time and tissues in a model
1441 reptile*. Physiological genomics, 2020. **52**(9): p. 423-434.
- 1442 168. Liu, S., et al., *Behavioral thermoregulation by reptile embryos promotes hatching success and synchronization*. Communications
1443 Biology, 2023. **6**(1): p. 848.

- 1444 169. Gárriz, A., et al., *Transcriptomic analysis of preovipositional embryonic arrest in a nonsquamate reptile (Chelonia mydas)*.
1445 *Molecular Ecology*, 2022. **31**(16): p. 4319-4331.
- 1446 170. Adams, D.M., et al., *Increasing hypoxia progressively slows early embryonic development in an oviparous reptile, the green turtle,*
1447 *Chelonia mydas*. *Royal Society Open Science*, 2022. **9**(8): p. 220709.
- 1448 171. Liu, W.L., et al., *Moderate climate warming scenarios during embryonic and post-embryonic stages benefit a cold-climate lizard*.
1449 *Functional Ecology*, 2022. **36**(5): p. 1137-1150.
- 1450 172. Babin, P., J. Cerdà, and E. Lubzens, *The fish oocyte: From basic studies to biotechnological applications*. 2007. 1-508.
- 1451 173. Polačik, M., et al., *Embryo ecology: Developmental synchrony and asynchrony in the embryonic development of wild annual fish*
1452 *populations*. *Ecology and Evolution*, 2021. **11**(9): p. 4945-4956.
- 1453 174. Kirchmaier, S., et al., *The Genomic and Genetic Toolbox of the Teleost Medaka (Oryzias latipes)*. *Genetics*, 2015. **199**(4): p.
1454 905-918.
- 1455 175. Feitosa, N.M., et al., *Brazilian silverside, Atherinella brasiliensis (Quoy & Gaimard, 1825) embryos as a test-species for marine fish*
1456 *ecotoxicological tests*. *PeerJ*, 2021. **9**: p. e11214.
- 1457 176. Dey, A., et al., *DNA repair genes play a variety of roles in the development of fish embryos*. *Frontiers in Cell and Developmental*
1458 *Biology*, 2023. **11**.
- 1459 177. Rafaella, et al., *Transgenic zebrafish (Danio rerio) as an emerging model system in ecotoxicology and toxicology: Historical review,*
1460 *recent advances, and trends*. *Science of The Total Environment*, 2022. **848**: p. 157665.
- 1461 178. Blechinger, S.R., et al., *Developmental toxicology of cadmium in living embryos of a stable transgenic zebrafish line*. *Environ*
1462 *Health Perspect*, 2002. **110**(10): p. 1041-6.
- 1463 179. Roosen-Runge, E., *Observations of the early development of the zebra fish, Brachydanio rerio*. *Anat Rec*, 1937. **70**(Suppl 1): p. 103.
- 1464 180. Howe, K., et al., *The zebrafish reference genome sequence and its relationship to the human genome*. *Nature*, 2013. **496**(7446): p.
1465 498-503.
- 1466 181. Bondue, T., et al., *The Zebrafish Embryo as a Model Organism for Testing mRNA-Based Therapeutics*. *Int J Mol Sci*, 2023. **24**(13).
- 1467 182. Ramdas Nair, A., et al., *Systematic Evaluation of the Effects of Toxicant Exposure on Survival in Zebrafish Embryos and Larvae*.
1468 *Curr Protoc*, 2021. **1**(9): p. e231.
- 1469 183. Blechinger, S.R., et al., *Developmental toxicology of cadmium in living embryos of a stable transgenic zebrafish line*. *Environmental*
1470 *health perspectives*, 2002. **110**(10): p. 1041-1046.
- 1471 184. Arteaga, C., et al., *The zebrafish embryo as a model to test protective effects of food antioxidant compounds*. *Molecules*, 2021. **26**(19):
1472 p. 5786.
- 1473 185. Bowley, G., et al., *Quantifying endothelial cell proliferation in the zebrafish embryo*. *F1000Res*, 2021. **10**: p. 1032.
- 1474 186. Hou, Y., et al., *Zebrafish as model organisms for toxicological evaluations in the field of food science*. *Compr Rev Food Sci Food*
1475 *Saf*, 2023. **22**(5): p. 3481-3505.
- 1476 187. Strähle, U., et al., *Zebrafish embryos as an alternative to animal experiments—A commentary on the definition of the onset of*
1477 *protected life stages in animal welfare regulations*. *Reproductive Toxicology*, 2012. **33**(2): p. 128-132.
- 1478 188. Rothenbücher, T.S.P., et al., *Zebrafish embryo as a replacement model for initial biocompatibility studies of biomaterials and drug*
1479 *delivery systems*. *Acta Biomaterialia*, 2019. **100**: p. 235-243.
- 1480 189. Canedo, A., et al., *Zebrafish (<i>Danio rerio</i>) meets bioethics: the 10Rs ethical principles in research*. *Ciência Animal Brasileira*,
1481 2022. **23**.
- 1482

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