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PERSPECTIVE

Magnitude and significance of the peak of early embryonic mortality

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Abstract
Embryonic development is of great importance because it determines congenital anomalies and influences their severity. However, little is known about the actual probabilities of success or failure and about the nature of early embryonic defects. Here, we propose that the analysis of embryonic mortality as a function of post-fertilization time provides a simple way to identify major defects. By reviewing the literature, we show that even small initial defects, e.g., spatial cellular asymmetries or irregularities in the timing of development, carry with them lethal effects in subsequent stages of embryogenesis. Although initially motivated by human study, in this contribution, we review the few embryonic mortality data available for farm animals and highlight zebrafish as a particularly suited organism for such a kind of study because embryogenesis can be followed from its very beginning and observed easily thanks to eggshell transparency. In line with the few other farm animals for which data are available, we provide empirical evidence that embryonic mortality in zebrafish has a prominent peak shortly after fertilization. Indeed, we show how subsequent mortality rates decay according to a power law, supporting the role of the early embryonic mortality peak as a screening process rapidly removing defective embryos.

Keywords Embryogenesis · Death rate · Post-fertilization age · Screening · Zebrafish

1 Introduction

The process by which any organism develops from a single-celled zygote to a multicellular organism is complex and therefore prone to error. Thus, embryonic development is of great importance because it determines congenital anomalies and influences their severity, and so it is crucial for ensuring the fitness of the organism. Whereas there is detailed qualitative knowledge of the successive steps of embryonic development, little is known about their

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probabilities of success or failure. However, and despite the lack of accurate data, it is in general accepted that natural mortality occurring during the early embryonic development may be high, as recognized in humans [1], in what is called the early embryonic mortality (hereafter, EEM) effect.

Far from being specific to humans, EEM seems to exist for all other species for which data are available [2, 3]. However, it is fair to acknowledge that very limited data on such early spikes of mortality are available so far (see below in section 2, entitled "Embryonic mortality in farm and laboratory animals"), and that the literature gives us almost no real clues for understanding the EEM effect. That is why we think it may be enlightening to begin establishing a parallel between living and technical systems to propose the conjecture that the EEM effect is essentially a screening process. A natural idea is that manufacturing defects are particularly crucial in components of complex systems which involve several steps and require high accuracy, as it is the case of embryonic development. In this context, we suggest that the study of embryonic mortality as a function of post-fertilization time provides a simple way to identify major defects and to quantify EEM. Then, in our search for a mechanistic basis, we describe how defects in early cell organization may carry lethal effects. Although initially motivated by human study, we do not try to give a detailed description of EEM for a single species but instead to focus on common features across animals for it appears that similar peaks occur in many species; in short, we study embryonic mortality as a global effect. We adopted such a comparative approach based on the view that the existence of the EEM effect can be better demonstrated by considering evidence drawn for a wide range of organisms. Notwithstanding, we also recognize that accurate data are needed and that these can be obtained primarily from experimental studies with model organisms. Thus, an important part of this contribution is devoted to discuss on the features that make zebrafish an ideal candidate among vertebrates for the study of EEM, and to provide comprehensive data on embryonic mortality as a function of time in this species.

1.1 Reliability engineering for the life scientist

In previous papers, a parallel has been introduced between living and technical systems [4–6]. From this perspective, and again taking human development as the starting point, three phases can be distinguished, including the following: (i) embryogenesis, from fertilization of the embryo to birth; (ii) from birth to 10 year-old there is the infant phase[1] [6–8], which is recognized as a wear-in phase and characterized by a decrease in mortality that follows a hyperbolic power law [5, 6]; and finally (iii) after the age of 10 years and until senescence and death, it comes a phase during which the death rate increases exponentially in accordance with the well-known Gompertz's law [9, 10]. In the language of reliability engineering, such a steady increase of the failure rate is known as a wear-out process.

[1] The medical definition of infant mortality is mortality of newborns under 1 year. This definition is mostly motivated by statistical convenience (a more detailed discussion can be found in [6], appendix A). From a biological perspective, it is more logical to include into the infant phase the whole age interval during which the death rate decreases which is 0–10 years. Recent research by the Global Burden of Disease studies [7, 8] follows a similar approach to ours by using neonatal (0–27 days), post-natal (28–365 days), and child (1–4 years) mortality rates to account for infant mortality, which is observed to decrease with age. Note also that the beginning of the death rate curve has no real signification for the following reason. The first point is for age less than 1 year. However, if we would take as first point the mortality rate for age less than 1 month, or less than 1 day, or less than 1 h, the rate would be at least 100 times higher.

Human age-specific curves for the three phases are given in Fig. 1. Whereas there are accurate data for phases 2 and 3, embryonic death rates—and especially the early embryonic rates—are subject to a number of uncertainties as emphasized in the review paper by Gavin Jarvis [1]. The main reason is the difficulty of detection of fertilization in the first 2 weeks (e.g., early hormonal dosage is subject to several external conditions). Similarly, early fetal deaths likely go unrecorded. For instance, in the USA, only fetal deaths at 20 or more weeks of gestation are recorded by states in the "National Vital Statistics System" [16]. Data from human in vitro fertilization studies report embryo mortality rates of 60–70% [17, 18]. Altogether, the available data suggest two comments: (i) despite the statistical uncertainties of human embryogenesis, because of its sheer magnitude (a sevenfold effect with respect to the base line), it is clear that there is a major mortality surge in the early phase of embryogenesis; and (ii) the EEM spike is not the only mortality peak to occur in the development of an organism.

Guided by the case of infant mortality which is basically a screening process through which individuals with defective organs are discarded [4], we propose the conjecture that the EEM effect is also a screening process, not of organs (at this stage there are none) but of the undifferentiated cells that appear in the first divisions of the cleavage stage.

1.2 From defects in embryonic cell organization to lethality

Mortality in early embryonic development may be accompanied by slight disturbances at the cellular level. Since we are interested in the earliest possible phase in which causes of mortality before birth may occur, we will focus on embryogenesis rather than organogenesis. Interestingly, investigations by two teams of researchers have shown that anomalies in initial cell organization are early signals of a compromised (and eventually lethal) development [19, 20]. A first effect may be due to spatial asymmetry in the cleavage phase. At this sense, Rideout et al. [19] have reported how a position asymmetry in 8-cell haddock embryos carries a lethal outcome in which almost all the embryos affected by such a defect die before hatching time. Of course, it would be of great interest to know the average time lag between the apparition of

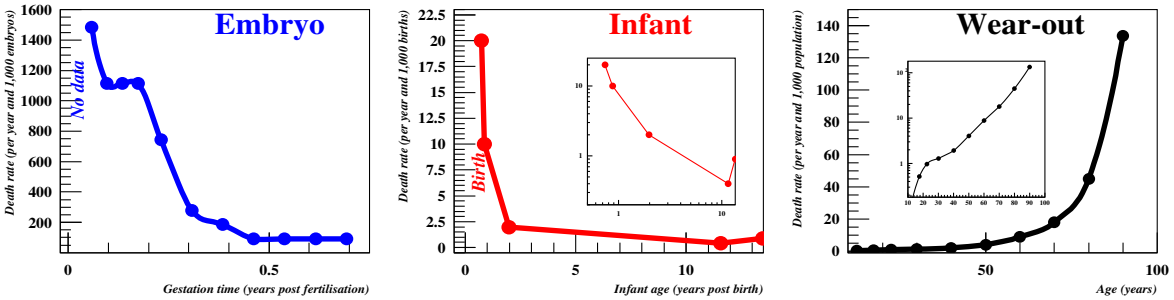

Fig. 1 Evolution of the death rate in the three phases of human development. In each phase, there is a huge peak. Whereas its existence is easy to understand in phase 2 and 3, the reason of the early embryonic mortality (EEM) peak remains a mystery. In phase 2, the decrease follows a hyperbolic power law, whereas in phase 3, the increase is exponential as described by Gompertz's law [9]. In phase 1, the exact shape of the curve is not yet known due to a lack of accuracy in empirical evidence, in particular in the first few weeks following fertilization. The expression "wear-out phase" in phase 3 is borrowed from reliability engineering; it is justified by the fact that for many functions, the best performance occurs between the age of 20 and 30 and then deteriorates steadily. Note that the maximum death rate of the senescence curve depends upon the oldest age group selected. A similar observation applies to the infant curve; in this case, inclusion of age groups close to birth would lift up the maximum of the death rate. The data for phase 1 are for the island of Kauai in Hawaii in the late 1950s [1, 11–13]. The data for phase 2 are for the USA in 1960 [14]. The data for phase 3 are for the USA in 2016 [15]. The insets of panel 2 and 3 are (log, log) and (lin, log), respectively

the defect (assuming a 20-min division time, the 8-cell phase would occur at around 60 min after fertilization) and the death of the embryo, but these data were not available. Note also that this defect is fairly similar to positional defects in a crystallized solid. A second effect may be attributable to asynchronous cell division. An observation based on 834 human embryos shows that timing irregularities in cell replication affect the subsequent development of the embryo [20]. It appears that for the 552 embryos that had a successful development (i.e., reached the blastocyst stage), the time spent in a 3-cell stage—indeed a deviation of the 2^{n-1} geometric sequence of cell division, being n the round of replication—was on average 0.6 h, whereas for the 282 unsuccessful developments, the time spent in a 3-cell stage was 1 h. All other parameters describing the timing of the divisions between successful and unsuccessful embryos were the same within a ± 10% margin. In other words, this observation suggests that a lack of synchronicity in cell divisions had a disturbing influence on embryonic development. It is interesting to note that in these two cases, the defects are not due to individual cells but rather to defects in their interaction.

1.3 A testable conjecture for the embryonic mortality peak

At this point, we have drawn a reasonable parallel between embryonic development and a manufacturing process in which early mortality is seen as a screening phase. Thus, during the fairly short time interval from fertilization to birth, the embryo has to undergo a succession of complex transformations. If initial steps are faulty, they may not provide an appropriate basis and starting point for subsequent transformations. We have also built a degree of confidence on this idea by providing mechanisms of uncoordinated cell division occurring shortly after fertilization that can lead to the death of the embryo. Now going further, we want to highlight that our idea of such a parallel between embryogenesis and a manufacturing process can be tested.

In reliability engineering, it is a common belief that, assuming identical technical equipment, manufacturing defects increase with the speed of the production process. The reason is that control procedures take time (even if they are automatic). Such controls improve overall reliability but at the cost of slowing down the process. In keeping with this logic, under the *caeteris paribus* clause for products of different complexity, a positive correlation between complexity and failure rate is expectable. Similarly, if we accept the conjecture that the EEM is a filtering process, one would expect a higher mortality rate for more complex organisms. To test this prediction in living organisms, we discuss the potential of a comparative approach between species that would take into account (i) the division times of undifferentiated cells (as a measure of the speed of the production process) and (ii) the degree of the complexity of the organism at birth.

For comparison purposes, organisms should ideally have similar initial cell division times. However, it turns out that there are great differences in early cell division times across animal species. For instance, it takes ~0.5 h for the nematode *Caenorhabditis elegans* [21] or zebrafish embryos [22], but longer times for mammal embryos (e.g., 14 h for mice [23], or 20 h for humans [24]). Yet, another difficulty is how to define species complexity. One approximation may be the diversity of specialized cell types in each organism [25], but these data are not available for most organisms yet. For the sake of simplicity, we retain the total number of cells at birth or hatching. This may be a fairly rough criterion but it has the advantage of being well defined. For instance, *C. elegans* has about 1000 cells whereas zebrafish have some 25,000 cells. Given that cell division times are similar for these two species, we could predict a lower hatching rate for zebrafish eggs than for *C. elegans* larvae

according to our conjecture. Once more data and better metrics become available for i and ii, it will be possible to test the conjecture.

Having established the basis of our conjecture and presented how the study of embryonic mortality as a function of time after fertilization provides a simple way of quantifying EEM, in the following sections, the paper will proceed as follows: (i) in addition to the human data already discussed in the "Introduction," we present embryonic mortality data for the few other animal cases that have been studied, and (ii) we present the design and main results of two experiments using zebrafish embryos, which in our opinion offer unique conditions for the exploration of the EEM peak.

2 Embryonic mortality in farm and laboratory animals

In this section, we review published data on embryonic mortality in the few cases—other than humans—in which age-specific data are available. Our aim is to provide evidence that EEM is a widespread effect. The two main sources from which data were retrieved are studies of farm animals (e.g., poultry or cattle) or of organisms used in laboratories. A note of caution in relation to terminology is introduced now as for humans (and more generally for mammals), a distinction is made between embryonic and fetal development, which is not the case in other animal groups (e.g., fish and birds). It is because of our aim to generalize that we will use the term "embryonic" for the whole process from fertilization to birth.

2.1 Embryonic mortality in poultry

We begin this subsection with the best case documenting EEM in avian species that we have found in the literature [26]. It is the case of chicken (*Gallus gallus domesticus*), in which the duration of embryonic development from fertilization to hatching is about 21 days [27]. Figure 2 depicts embryonic mortality as a function of post-fertilization age from data given by Peñuela et al. [26]. In that study, the numbers of deaths in each age interval were obtained in two ways: (i) by candling (i.e., screening the egg with the help of a light source) and (ii) opening of unhatched eggs after the 21-day follow-up period. In both cases, the day of death was established according to the parameters of Hamburger and Hamilton for every day of incubation [28]. At that point, the distinction between non-fertilized and fertilized eggs could be made easily: any egg that experienced embryonic development, no matter how small, was considered fertilized. In support of the existence of the EEM effect, our analysis on the data retrieved from [26] evidences that there is an early spike of mortality followed by a decrease in death rate up until the middle of the incubation period. Then, there is another maximum just before hatching. It has several possible, not self-excluding, explanations. On one hand, it was observed by Hutt [29] that one half of all chick embryos which die between day 18 and 20 were in an abnormal position which did not give them access to the air cell which is on the blunt tip of the egg. On the other hand, the lungs of chicks start to work shortly before hatching; previously the oxygen absorbed into the egg through the shell was transferred to the embryo through a network of thin blood vessels constituting a kind of rudimentary lung called the allantois. If the amount of oxygen delivered is insufficient, breaking the shell becomes impossible. Furthermore, because for chicks (and more generally for all avian species whose eggs have a fairly hard shell) breaking the shell is a very challenging task, one is not surprised to see a high pre-hatching mortality peak. Similar results have been reported for other avian species, such as pigeons, doves, ducks, grouse, pheasants, quails [30], or turkeys [31].

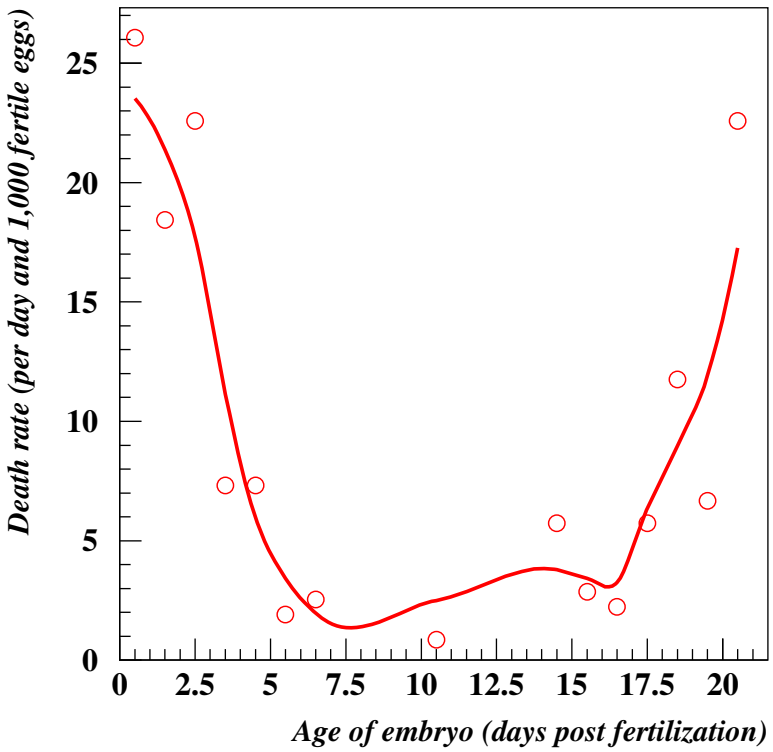

Fig. 2 Embryonic death rate in chicken. Death rate was computed as the number of deaths in a given age interval divided by initial number of embryos. A total of 3240 eggs were examined, and there were a total of 471 embryonic deaths, which give a hatching rate of 82%. Of these deaths, 57% occurred during the first week. Older age of the female increased death rates but did not change the shape of the curve. Malposition of the embryo inside the egg was a major cause of pre-hatching death. Curve smoothing was accomplished by means of polynomial interpolation. Original data taken from [26]

2.2 Embryonic mortality in cattle

The widespread use of artificial insemination in cattle allows accurate determination of the time of fertilization. Among these animals, the case of cow is illustrative for the EEM effect [2, 32]. Figure 3 has been built up on the data from [2] and shows that the mortality peak in this species occurs shortly after fertilization. In contrast with Fig. 2, there is no late maximum. This cannot be seen in cows because the graph does not extend to the end of gestation, which occurs at around week 40. Interestingly, Wathes and Diskin [33] have showed that the major component of embryo loss occurs early during pregnancy (from insemination to week 8, the pregnancy losses are about 50% of initial pregnancies), whereas late embryo losses are numerically much smaller (between week 8 and the end of pregnancy are only 5%).

Evidence of EEM is also reported in other cattle animals [32], but data are less detailed than for cows, with only the percentages of embryo deaths occurring in the first third of pregnancy reported (e.g., 25% in ewe, 40% in sow, and 40% in mare; in all cases, percentages expressed over the number of successful inseminations).

2.3 Embryonic mortality in lab animals

Regrettably, almost no mortality data as a function of embryonic age could be found in the literature. To our knowledge, the only available case concerns the European perch (*Perca fluviatilis*) [34]. As we will show below, it reveals a huge initial peak. There is also some

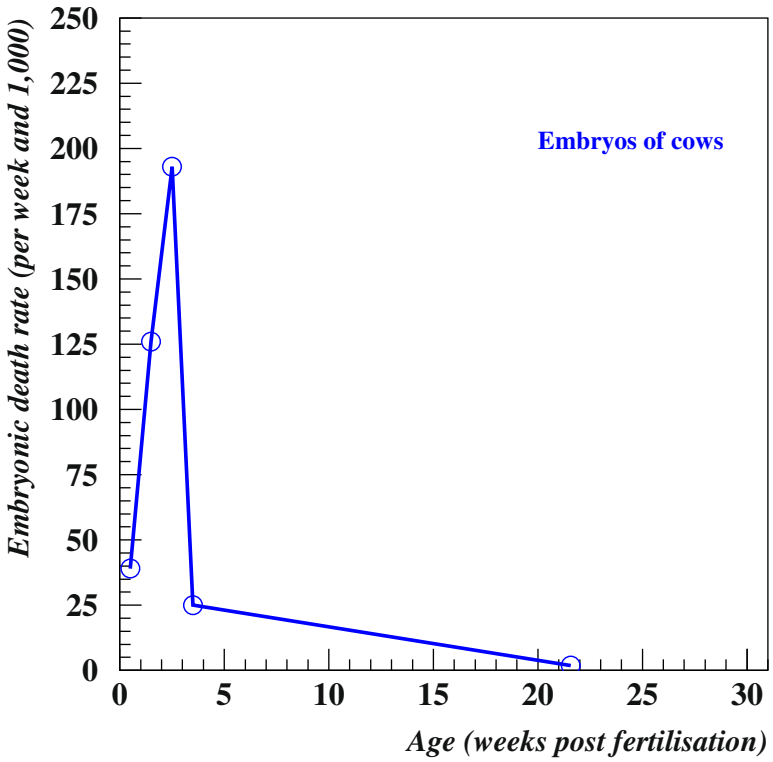

Fig. 3 Embryonic death rate in cow. The graph gives death rates per week of age and 1000 successful inseminations. The study involved 63 cows, and there were 35 embryonic deaths, which give a calving rate of 55%. The maximum occurred in the third week, i.e., 15 to 21 days after fertilization. The whole pregnancy lasts about 280 days. Dots were connected through straight lines, instead of a smoothing procedure, because of the low number of observations available. Data from [2, 32]

support in favor of the EEM effect from recent experiments with the African clawed frog (*Xenopus laevis*) and zebrafish (*Danio rerio*) by Uchida et al. [35], but the mortality studied through their respective embryonic development was not age, but stage specific. Interestingly, the period of high embryonic death was observed around the gastrula period in both zebrafish and *Xenopus*. Although data are still scarce, these results are promising and serve to emphasize the importance of comparative studies across animals to ensure a broad range of validity of the results. It also points to zebrafish as a suitable organism for the accurate characterization of post-fertilization age-specific embryonic mortality. This issue was addressed experimentally in the study detailed in the next section.

3 Exploration of the mortality of zebrafish embryos

Zebrafish offer ideal conditions for the exploration of embryonic mortality for a number of reasons:

(i) As for most fish, zebrafish eggs are fertilized outside of the body of the female. Therefore, in contrast to many other organisms, the embryo can be observed from the very moment of fertilization to hatching.
(ii) Eggs are transparent, so the development of the embryo can be easily observed and monitored.
(iii) After death of the embryo, the eggs become opaque and therefore can be easily identified

(iv) The embryonic phase lasts about 3–4 days (depending on temperature), which is a convenient duration to carry out the required observations.
(v) Finally, as a model organism actively studied in genetic research, zebrafish are raised in many university laboratories.

Here we took advantage of these features to experimentally get insights on the EEM effect in this species. For this purpose, we carried out two laboratory experiments (hereafter, Experiment 1 and Experiment 2). The goal of both experiments was to measure the mortality rate of zebrafish-fertilized embryos as a function of post-fertilization age. Notwithstanding, each experiment was oriented to explore a different aspect concerning the EEM effect. Experiment 1 was performed first and devoted to explore replicability (i.e., whether EEM was conspicuous and consistent across independent replicate cohorts of zebrafish embryos). In this first experiment, much effort was put into the individualized monitoring of the developmental progress in the embryos to lay the foundation for a suitable methodology for further experiments. Once this was achieved, Experiment 2 was oriented to obtain a greater accuracy in the characterization of mortality as a function of post-fertilization age and to mathematically characterize the shape of the embryonic peak decay in zebrafish.

3.1 Methodological aspects

3.1.1 Experiment 1

Experiment 1 was performed during the summer of 2019 at the Institute of Physics of the Chinese Academy of Science in Beijing (China). Zebrafish eggs were produced by standard breeding techniques at the Life Science Department of Peking University. We split the experiment in four trials from independent breeding boxes (4 male-female pairs on average per box) in which the starting numbers for each of the four trials ranged 277–672 fertilized embryos. For the ease of monitoring, eggs from particular trials were produced in successive batches (i.e., cohorts) so that the largest trial in our experiment numbered 770 fertilized embryos. The total number of fertilized embryos monitored in Experiment 1 was 1673.

Experimental steps are summarized in Table 1. Firstly, after breeding, we separated the fertilized from the unfertilized embryos as early as possible. The fertilized eggs can be easily distinguished from the non-fertilized after they reach the 2-cell stage (a double hump is observed in fertilized embryos; see Fig. 4), which occurs about 0.75 h after egg production [36]. This moment is critical for monitoring because cell division progresses rapidly in the following hours. At each division, the new cells become smaller because after division, they are not given enough time for further growth. When there are ≥ 32 cells, they form a large hump in which individual cells can no longer be seen and which is not very different from the single humps of unfertilized eggs (see Fig. 5, panel 2). Thus, the separation of unfertilized eggs should best be done between 1 and 2 h post-fertilization (hereafter, hpf).[2]

One way to test whether the separation was done satisfactorily is to collect the discarded eggs and to see if all of them become opaque after 24 h. In our experiment, we classified an

[2] As the eggs must be examined one by one, a single operator can hardly treat more than 200 eggs per hour. For larger samples, the solution is to increase the number of operators or/and produce the eggs in successive batches by removing the separations in the breeding boxes with time lags of about 1 h. We have been using two operators and 1-h time lags between successive batches.

egg as fertilized only if 2 or 4 humps could be identified clearly. This is a conservative approach that may underestimate the numbers of fertilized embryos (i.e., some eggs are rejected even though they may have been fertilized). Such a drastic selection is necessary because it is crucial not to include any unfertilized egg, as this would artificially inflate initial death rates. The fact that ca. 95% of our discarded eggs became opaque after 24 h shows that the procedure worked fairly well.

All fertilized embryos were incubated at 23 °C in 96-well plates during the experiment. Although for zebrafish a temperature of 28 °C is considered optimum [36], we choose a lower temperature within the optimum range. At this sense, Schirone and Gross [37] have reported that embryos appear to develop normally at 23 °C, and this temperature was closer to room temperature in the lab so that heat shocks during manipulation (i.e., every time embryos are taken out of the incubator for observation) were avoided. During monitoring, dead embryos were identified by opacity, as primary diagnostic criterion. However, there may be a time lag[3] between the moment when an embryo dies and the moment when the egg becomes opaque. Therefore, prior to (and independently from) egg opacification, one can use the following two complementary criteria: (i) the shutdown of further development and (ii) embryo's immobility. With respect to the identification of deaths and the usage of the three diagnostic criteria, we can recognize 3 phases:

(i) The cleavage phase, which lasts about 2 h and during which it is possible to count the number of cells in the embryo. Due to the short duration of this phase, the abovementioned time lag may be critical, and so it is convenient to employ the "no development" criterion to assess embryo death. We did not see any stoppage in development in this phase.[4]
(ii) After that, the embryo assumes a shape on which changes are not easy to identify. This is illustrated in Fig. 5. At this stage, the most frequent defect is the lack of a thin line around the ball of yolk. Observation shows that this defect leads to death and opacity within a few hours.
(iii) Once the embryo assumes the shape of a fish larva, the development becomes easier to follow. The heart begins to beat at around 25 hpf and the flow of blood becomes gradually more visible particularly in the yolk sac and in the tail. In this phase, dead embryos can be clearly identified and counted.

With these criteria at hand, we identified and counted the number of dead embryos in the course of the experiment for each of the four trials. These numbers were used to (i) compute mortality rates at different age during embryonic development, (ii) to compare the survival curves of the developing embryos among the four experimental trials, and (iii) to cross-check the distributions of the age at which embryo death was observed between trials. Survival curves were estimated using Kaplan-Meier [38] method and compared by log-rank test by means of the functions within the "survival" [39] and "survminer" [40] packages in R [41]. Pairwise comparisons of the time distributions of the number of dead embryos by trial were tested by means of Kolmogorov-Smirnov tests performed in R as well.

[3] Our observations suggest that this time lag increases in the course of egg development.
[4] No embryo in the cleavage stage was ever seen arrested in the course of our experiments. At first sight, this may suggest that in this age group, the death rate is very low. It is certainly low but one must take into account that this initial cleavage phase lasts less than 2 h; thus, the probability of seeing a death is naturally much lower than for a phase that lasts several hours.

Table 1 Operations for measuring the number of deaths in zebrafish embryos in Experiment 1

Step	Time (hpf)	Device	Operation	Purpose
1	(0, 0.2)	Breeding boxes	Production of eggs (some 100–150 per batch)	
2	(0.2–1.5)	Stereomic (20×)	Examination of all embryos	Separation of embryos with 1 hump from those with ≥ 2
3	(1.5–3)	96-well plates	Repartition: 1 embryo per well	Monitoring embryo development individually
4	(3, 12)	Light pad + magnifying glass (10x)	Observation of all embryos	Identification of opaque embryos
5	After 12	Stereomic. (20x)	Identification of late comers	Checking for development arrestment
6	After 24	Stereomic. (20x)	Examination of all embryos	Checking for heart beating
7	After 60	Light pad	Identification of hatched embryos	

Notes: "hpf" means hours post fertilization. Two modes of observation were alternated. The light pad surveys were fast (for a 96-well plate, it took 5 min instead of 30 min for a microscopic observation of all the eggs), and therefore, they could be repeated often. However, microscopic examination was necessary to identify the embryos that were on hold and to see whether or not their heart was still beating. The identification of laggards gives a first signal that something is wrong. It can lead to death or to recovery

3.1.2 Experiment 2

Experiment 2 aimed to increase both the accuracy of the mortality rate measurements and the number of observations throughout the development of zebrafish embryos, in order to mathematically analyze the shape of the decay in embryo mortality. To do this, we increased the initial number of embryos in a single trial and changed the monitoring procedure. The experiment was carried out at the Biology Institute of Paris-Seine (France).

Unlike the previous experiment, in Experiment 2, the embryos were not individually monitored in multi-well plates, but instead time-lapse pictures were taken of a single Petri dish with ca. 2000 zebrafish embryos at 20 min intervals during 3 days. Embryo mortality in this experiment was defined according to the opacity criterion. Therefore, the procedure excludes cases where development is stopped without the embryo becoming opaque. Although such cases exist, data from Experiment 1 showed that they are rare (ca. 1%). The loss of these embryos is compensated for by the advantages of the new procedure, which include (i) being able to monitor a larger number of embryos, (ii) with a greater frequency between observations, and (iii) being almost completely automatic, except for the selection of the fertilized eggs at the beginning of the experiment.

Mortality rates were computed with the same definition as for infant mortality rates (i.e., number of deaths in a given age interval divided by number of initial embryos) and were used to analyze how the mortality rate changes as a function of age over embryonic development. For this purpose, we fit the mortality rate values with a general equation describing a power law function ($y \sim A/x^{\gamma}$). This function was selected because it is illustrative of a screening process and is known to fit age-dependent infant mortality rate curves in a wide range of animals, including zebrafish [5]. Fitting was performed by nonlinear least-squares estimates of

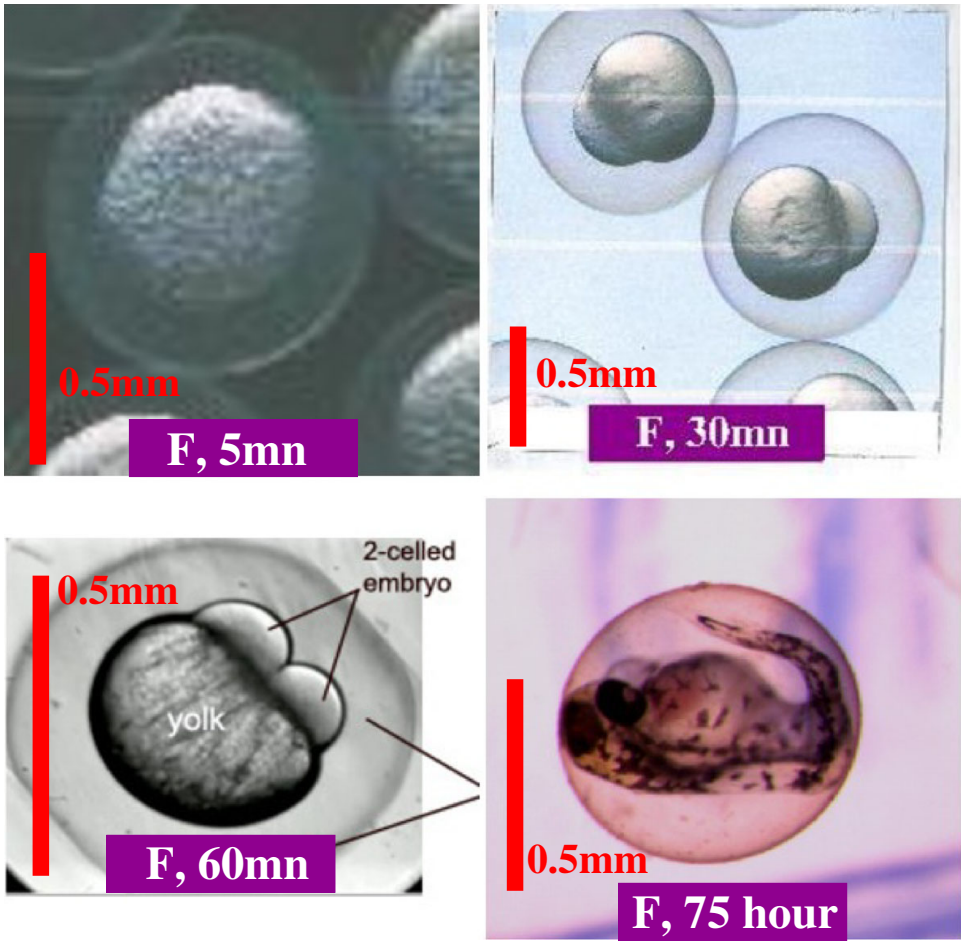

Fig. 4 Successive steps in the development of fertilized zebrafish embryos. A critical step occurring about 1 h after fertilization is the apparition of a second hump (picture 3), which results from the first division of the initial embryo cell. The last picture shows the embryo shortly before hatching. All pictures (except for panel 3) were taken at the Biology Institute of Paris-Seine

A and γ parameters by means of the *nls* function from the "base" package and supplementary functions form the "nlstools" package [42] in R. The correlation coefficient R^2 was used as a measure of goodness of fit.

3.2 Experimental results for embryonic mortality rates in zebrafish

3.2.1 Experiment 1

The curves that summarize the results of the four experimental trials for studying the mortality rates of zebrafish embryos as a function of age are presented in Fig. 6. Notably, and in spite of the observed differences in mortality levels (i.e., survival analysis based on the log-rank test revealed the existence of significant differences between the four trials with p value < 0.001), the shapes of the four curves were similar. In all the four trials, the number of dead embryos started to increase shortly after fertilization and reached a maximum at about 10–20 hpf. Pairwise comparisons through Kolmogorov-Smirnov tests revealed that the distributions of the timing of embryonic deaths of the four trials behaved in the same manner (p value for all-pair comparisons > 0.05). Overall, our results show that the peak of mortality in zebrafish occurred within the first 15% of the 75–80 h of embryogenesis and was about 50 times higher than the low plateau that followed.

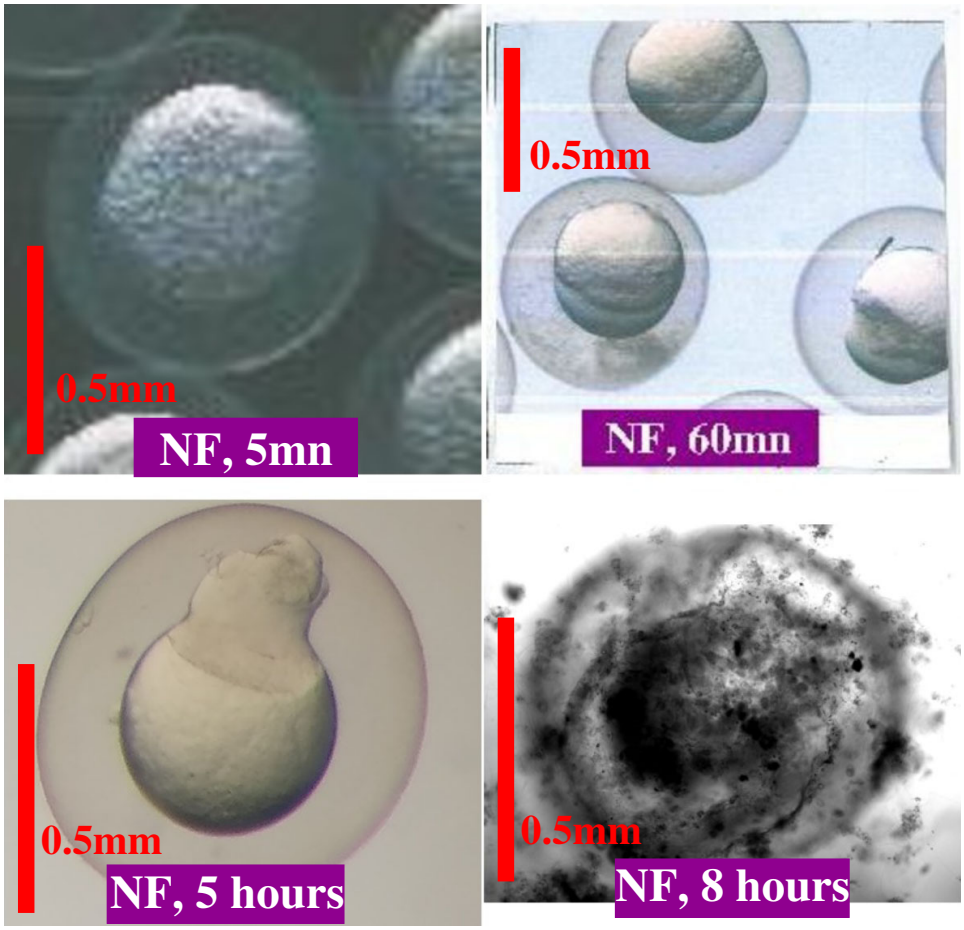

Fig. 5 Successive steps in the development of unfertilized zebrafish embryos. The unfertilized eggs can be obtained directly from a female by a gentle massage on its belly. After that, the process starts when the eggs are released in water. In fertilized as well as unfertilized eggs, one sees the apparition of a first hump. Then, whereas the fertilized eggs develop a second hump as shown in the previous figure, in unfertilized eggs, the single hump continues to swell while at the same time taking fairly weird shapes as shown in the third picture (taken some 5 h after release in water). In a process that starts some 8 h after release, all non-fertilized eggs will turn opaque as shown in the last picture. Pictures were taken at the Institut de Biologie de Paris-Seine by Alex Bois and at the Beijing Normal University by Yi Zhang

Although the observed replicability provides strong support for the EEM effect, one may still wonder what is the main factor causing variability among cohorts. The comparison of the four curves strongly suggests that the characteristics of the parents are essential. On average in our experiments, some 4 male-female pairs contributed to the production of the eggs. Such a low number of parents may be not enough to achieve homogenization by mixing. In fact, due probably to the young age of some of the parents (meaning that they have not yet been used for reproduction), the 4th trial involved a high percentage of defective eggs which led to a huge screening process. The poor quality of these embryos was apparent by the high initial number of eggs in bad shape (eggs of irregular shape, broken shells, eggs containing black particles). In such a case, if the first day acts as a filter, one expects a high early mortality. It is remarkable that after the first 24 hpf, the mortality rate of this trial fell as rapidly as for normal embryos.

3.2.2 Experiment 2

Experiment 2 confirmed our previous results with a completely different procedure, with a clear peak of the mortality rate in zebrafish embryos observed at about 12 hpf (Fig. 7). The

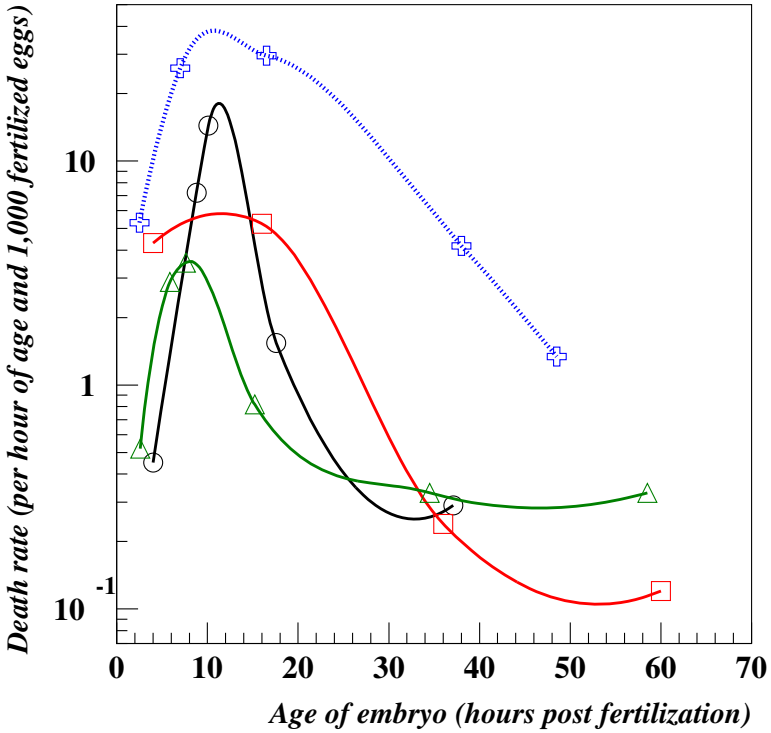

Fig. 6 Embryonic mortality rates for fertilized zebrafish embryos in Experiment 1. Each curve is for a separate series of observations. The numbers of fertilized embryos were as follows: circles, $n = 277$; squares, $n = 345$, triangles, $n = 379$, and crosses, $n = 672$. Note that the vertical scale is logarithmic and that age intervals were merged to avoid intervals with zero deaths when necessary. Curve smoothing was accomplished by means of polynomial interpolation

higher resolution of Experiment 2 with respect to Experiment 1 allowed us attempting the mathematical characterization of the drop in the mortality rate after the peak of mortality observed at the onset of ontogeny.

Taking as a guide the case of infant mortality, which is interpreted as a screening process [4], here we have proposed the conjecture that the EEM effect is a process of that same kind. As the birth peak decay is known to be hyperbolic [5], a similar shape for the EEM fall would give further confidence in a parallel between the two phenomena. Our analyses showed that the distribution of mortality rates as a function of post-fertilization age fitted well with a power-law decay model right after the spike, as evidenced by R^2 goodness-of-fit (0.83). The estimate for the power law exponent (γ) was equal to 2.65 ± 0.95. If compared to the results presented in Bois et al. [5] for a 50-day post-hatching period, which showed a power-law decrease with exponent 4.5, it arises that screening processes for embryo and larvae follow fairly similar patterns. This similarity suggests a kind of defect that is lethal within a short time in both cases, i.e., a quick elimination of defective embryos or larvae. Worthy to mention, this does not mean that mortality rates are similar for both phases; even at the peak, it is slightly higher for the post-hatching period than for embryonic development, but that the subsequent drop in mortality to the plateau follows a similar pattern.

4 Conclusions

By establishing a parallel between the lifetimes of technical and living systems [4–6], we have proposed the conjecture that at the beginning of embryogenesis of any type of organism, there

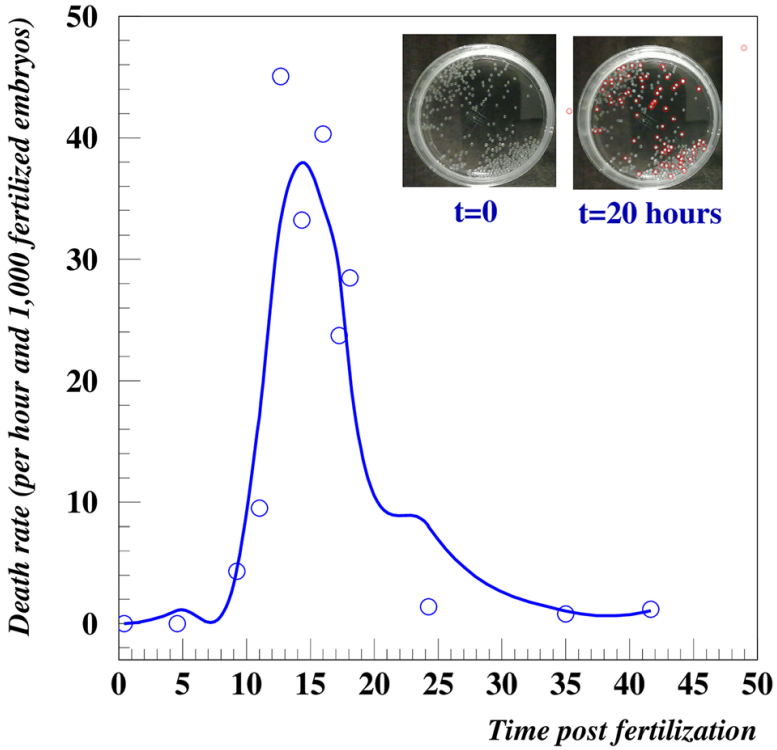

Fig. 7 Embryonic mortality rates for fertilized zebrafish embryos in Experiment 2. The insets represent pictures taken at the start of the experiment ($t = 0$) and at 20 hpf of the Petri dish in which zebrafish embryos were settled. Opaque (dead) embryos at 20 h are highlighted in red. Curve smoothing was accomplished by means of sequential moving average procedure and polynomial interpolation. This was necessary because a polynomial interpolation alone would have resulted in wild artificial extrema

is a filtering process that operates as a purge of the defective embryos and leads to the EEM peak. For such a surge of the mortality rate, there can be two sources: (i) the initial elements (i.e., oocyte and sperm cell) may be defective (see Fig. 8, which extends the mortality phases to include the still unexplored pre-embryonic phase), and (ii) subsequent defects may arise either in the process of fertilization or in the early stages of the development, of which we have given two examples related to undifferentiated cell interactions [12, 13]. Good quality embryos must exhibit appropriate kinetics and synchrony of division. Otherwise, they are quickly eliminated at a particular checkpoint early during development.

The EEM seems to be a widespread effect among animals, from mammals [1–3, 32, 33], to birds [26, 27, 30, 31], and fish [34, 35]. Our review of the existing literature and the new data provided in this contribution support this idea. However, limited data on embryonic mortality are available so far. Those recorded in our experiments for zebrafish embryos may be the most accurate ever produced. There are, at least, three reasons for that: (i) the experiments were especially designed for the observation of EEM as a function of age (whereas previous experiments with farm animals had mostly an economic goal), (ii) they involved samples of over 1500 embryos (whereas it is difficult to have samples of this size for bigger animals), and (iii) trial replicates served as internal quality checks on how the experiments were performed.

However, among the few previous studies, we would like to highlight that of Uchida et al. [35], for several reasons. One reason is that these authors explored embryonic mortality patterns in zebrafish embryos as a function of stage development, but also those in chicken and the African clawed frog. Thus, a comparison across different taxa in EEM was outlined. Interestingly, their results for zebrafish were paralleled by similar results for chicken and African frogs. Another reason is that these authors provide a methodology that supports the

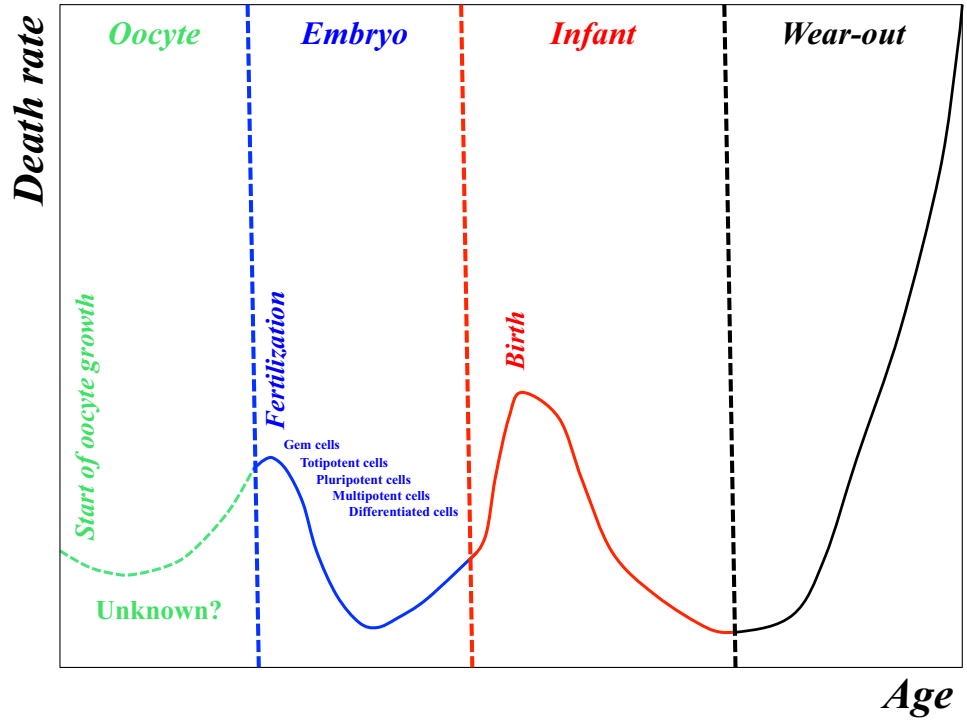

Fig. 8 Evolution of the death rate in the four phases of development. This figure illustrates a conjecture, which generalizes Fig.1 in two ways. Firstly, it introduces an initial phase corresponding to the "manufacturing" of the spermatozoa and oocytes, respectively, containing the male and female germ cells. Secondly, this process in four phases is presumed to apply to a broad spectrum of species from fish to mammals and birds. However, it excludes organisms whose development involves metamorphosis through successive stages (e.g., larva, pupa, imago), which follow a more complicated process

idea of the role of EEM as a screening process. By submitting embryos to temporary shocks (including temperature shifts, UV exposure chemical inhibitors), it was found that development is more susceptible to these shocks in early than in late stages of embryogenesis, in the sense that further development is more affected by early than by late shocks. If returning to the parallelization between technical devices and biological systems, one can interpret some of those temporary shocks, for instance shocks in temperature, as changes in the conditions of "production" (without any effect on the "design").

The EEM effect can be studied in much better conditions in fish than in mammals or birds because in the former fertilization occurs outside the body of the female and can be monitored from the very beginning. Zebrafish eggs have the additional advantage that their shells are transparent, a feature that is not common to all fish. Other additional features of the embryo in zebrafish have been highlighted to justify our choice as experimental model species. The measurements presented in the two experiments carried out in this study show that there is a huge mortality peak at the beginning of the embryonic phase in zebrafish embryos. This observation comes in confirmation of previous observations made for zebrafish [35] and another fish species, the European perch, the only previous dataset with available age-specific embryonic mortality rates [34].[5] Both studies are consistent in showing the existence of the EEM effect. Further, our results from Experiment 2 support that embryonic mortality in

[5] Based on her recent research at the Institute of Marine Research in Bergen, Dr. Maud Alix has found that for cod (*Gadus morhua*), there is also clear evidence for an early embryonic mortality peak (personal communication, results to be published shortly). As mentioned above, the results published for zebrafish by Yui Uchida et al. [35] go in the same direction, but the effect is less conspicuous due to a small sample of embryos (n = 72).

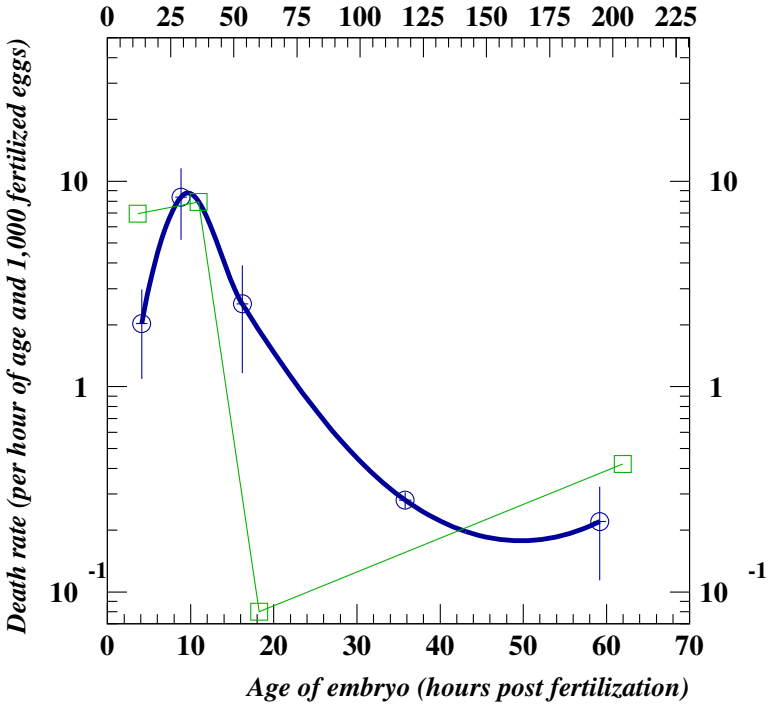

Fig. 9 Average embryonic mortality rates for zebrafish (circles, lower horizontal scale) and European perch (squares, upper horizontal scale, data from [30]). Error bars are ± σ, which defines the confidence intervals with a probability level of 0.66. Curve smoothing for zebrafish data was accomplished by means of polynomial interpolation. Note that drawing a smooth line through the sharp peak in the European perch was hardly possible due to low number of available data points, which is why we drew straight connecting segments

zebrafish decreases in a fashion similar to infant mortality, so it can be interpreted as a screening process [4]. Taking inspiration again from the birth process in which the screening process (i.e., the post-yolk filtering in the case of zebrafish [5]) is triggered by an environment change, we can infer that here too, there is an environment change when the embryo becomes autonomous and does no longer rely on the support of the mother. For instance, a partially defective function may have been acceptable as long as it could be propped up through interaction with the mother. Once released in the water, the fertilized embryos must rely on their own resources. The successful completion of the second hump seems to signal that the zebrafish embryo has passed this first screening.

A final issue we have pointed out in this study is the need for comparative studies between species. Naturally, one may be tempted to extrapolate the results shown in our experiments to other organisms, including humans. At this sense, age-specific mortality rates during the embryonic development phase are required for a proper comparison. We have seen that the characteristics of zebrafish embryos offer advantages in making these measurements accurately, but we are still far from having them for many other organisms. A very preliminary attempt with the data already available shows that the amplitude of the age-specific mortality curves is similar for the zebrafish (from Experiment 1) and for the European perch [34] (see Fig. 9), which suggests that embryonic death rates may be fairly robust despite size differences (recall that perch is a much bigger fish than zebrafish), at least within animal classes. We do not yet know if this can be generalized to include animals from other classes, but data on infant mortality show different rates among the major groups of animals [5]. Once more age-specific data become available, a matter for future study will be to analyze whether there is a correlation between EEM rates and the slopes of regression lines of the subsequent mortality

rates (which are identical to the γ exponents of power-law falls) across taxa. It would be expected that higher initial mortality rates lead to higher power law exponents. This has been called the rate-exponent rule for the case of infant mortality [4, 5]. Yet, a further step when this rule is confirmed for embryonic mortality will be to understand why the initial mortality rate differs among taxa. In this contribution, we have adduced that the mortality rate could be positively correlated with the number of diversity of defects during embryonic development that is to say the complexity of the organism. For the sake of simplicity, we retain the number of cells at birth as a measure of complexity as there are data available for many species. Nevertheless, we note that cell number at birth remains a very crude estimate of complexity and hope that better measures will be further developed to test this conjecture. Overall, this prediction would allow the role of EEM effect to be tested as a global screening process across animal species.

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Compliance with ethical standards

According to the EU Directive 2010/63/EU on the protection of animals used for scientific purposes, early life-stages of zebrafish are not protected as animals until the stage of being capable of independent feeding (5 days post fertilization). For experiments involving only embryos no prior approval was required.

Conflict of interest The authors declare that they have no conflict of interest.

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