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Evaluation of temperature- and ethanol-related developmental degree variations by a new scoring system (FETAX-score) applicable to Frog Embryo Teratogenicity Assay: Xenopus

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ABSTRACT

The aim of the present work is to propose a new quantitative assessment method (FETAX-score) for determining the degree of *Xenopus laevis* embryo development intended for use in embryotoxicity studies. Inspired by a similar scoring system used to evaluate developmental delays (young-for-age phenotypes) in rat embryos cultured *in vitro,* the FETAX-score was established by considering seven morphological features (head, naris, mouth, lower jaw, tentacles, intestine, anus) that are easily evaluable in tadpoles during the late stages of development at the conclusion of the test. Given that *X. laevis* development is temperature-dependent and that temperatures below 14°C and above 26°C are teratogenic, the FETAX-score was tested in embryos maintained at 17, 20, 23 and 26°C. No abnormalities were observed in any group, while the total score was temperature-related, suggesting that the FETAX-score is sensitive to moderate distress that does not influence general morphology. Intestine and anus were the least sensitive structures to temperature variations. To assess the applicability of the FETAX-score in developmental toxicological studies, we evaluated FETAX-score in tadpoles exposed during the morphogenetic period to Ethanol (Eth) at concentrations of 0, 0.25, 0.5, 1, 1.5, and 2 % v/v. Gross malformations were observed only in tadpoles from the Eth 2 % group. By contrast, data analysis of the other Eth groups showed dose-related reductions in the FETAX-score. Tentacles were the most sensitive structures to Eth-related delays. These results support the use of the FETAX-score to quantitatively assess developmental deviations in FETAX embryotoxicity studies.

1. Introduction

In developmental toxicology, both epidemiological and experimental studies consider embryo/foetal lethality, gross malformations or alterations in the rate of development (such as delays or overgrowth, defined as young/old-for-age and small/large-for-age) as key endpoints, useful for risk assessment. Consequently, in experimental developmental toxicology, it is important to carefully assess the exact developmental stage at the end of the test as part of developmental and reproductive toxicology. Measuring overall embryonic development helps standardize evaluation and can be applied to identify any deviations in growth from the normal developmental rate caused by exposure. Researchers have defined stages of embryonic development in humans and various animal models (Jirasek, 1978 for man [1]; Hamburger and Hamilton, 1951 for chick [2]; Theiler, 1972 for mouse [3]; Edwards, 1968 for rat and rabbits [4] and Nieuwkoop and Faber, 1956 for the amphibian *X. laevis* [5]). In absence of easily detectable quantitative parameters for young/old-for-age assessment, different scoring methods were designed. Concerning alternative embryotoxicity tests, developmental degree scoring systems have been proposed in zebrafish [6,7] and in postimplantation rodents cultured *in vitro* (WEC (whole embryo culture) methodology) [8].

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Recently, we described a refined method (R-FETAX, Refined-Frog Embryo Teratogenicity Assay: *Xenopus*) as sensitive as WEC for developmental toxicological purposes [9,10]. R-FETAX is a promising alternative test to evaluate a spectrum of complex developmental disorders induced by chemicals, because it includes a windowed exposure period, covering specific developmental stages predictive for different outcomes: pre-organogenetic period, organogenetic period (sensitive for teratogenicity, evaluable by morphological examination techniques and functional tests), and spontaneous swimming acquisition period (sensitive for neuro-behavioural disorders, evaluable by functional tests) [9]. Like traditional FETAX, R-FETAX procedures are intended to end before Nieuwkoop and Faber (NF) stage 47 [5] considered the first stage included in EU Directive 2010/63/EU on the protection of animals used for scientific purposes [11]. According to NF staging criteria, the description of tadpole developmental stages at the end of the test considers a few simple main parameters, visible at NF stages 45, 46 and 47: NF stage 45 is characterized by "operculum partly covering gills with edge still straight, intestine spiralized in ventral aspects showing 1.5 revolutions"; NF stage 46 tadpoles show "2 edge of operculum becoming convex, xantophores appearing on eye and abdomen, intestine showing 2–2.5 revolutions, hindlimb bud visible for the first time"; NF stage 47 is described with "tentacles larger, edge of operculum forming quarter of a circle, xantophores forming opaque layer on abdomen, intestine showing 2.5–3.5 revolutions" [5,12]. Intestine coiling is the most evident and measurable parameter and therefore is considered crucial to evaluate stages 41–46 both in *X. laevis* and other *Xenopus* species [13]. Consequently, in our laboratory, a 2–2.5 turns coiled intestine was in the past considered representative of NF stage 46, normally reached at the end of R-FETAX [14,15]. However, we have often observed that intestine morphology, at the end of R-FETAX, may not align with the characteristics of other parameters classically assigned to NF stage 46. This highlighted the need for a quantitative method to precisely assess alterations in the rate of development in FETAX tadpoles.

The objectives of the present work were to assess a quantitative scoring system (FETAX-score) appliable to *X. laevis* tadpole evaluation. Temperature-related modifications in the time to reach specific developmental milestones were induced to test the FETAX-score's applicability. Finally, the FETAX-score was introduced to evaluate Ethanol (Eth)-related developmental delays reported in humans at not teratogenic exposure levels. Based on our previously published data, selected Eth concentrations were 0–0.1–0.5–1–1.5 % v/v, while Eth 2 % v/v was excluded from scoring .

2. Methods

2.1. Scoring system methodology

The proposed scoring system (FETAX-score) was designed observing the normal development of unexposed embryos maintained under standard controlled experimental conditions and considering the normal table of *X. laevis* development: seven morphological features (head, naris, mouth, lower jaw, tentacles, intestine, anus) were selected as representative of developmental stages defined by Nieuwkoop and Faber [5,12] as stages NF 40–47. A score ranging from 0 to 7 was assigned for each feature, based on simple morphological characteristics. The sum of the scores for each parameter provided the individual overall morphological score (total score).

2.2. R-FETAX methodology

Adults of *Xenopus laevis* (Nasco, USA) were maintained in an automatic breeding system (TecnoPlus, Techniplast, Italia) with controlled conditions (T = 20±2°C; pH = 7.5±0.5; Conductivity = 1000±100 μS), 12 h light/dark cycle (light from 7:00AM to 7:00 PM) and fed with a semisynthetic diet twice a week (XE40 by Mucedola; Settimo Milanese, Italy). Embryos, obtained from overnight natural mating of pairs, were dejelled L-cysteine 2.25 % dissolved in FETAX solution, selected and maintained at 23°C in Petri dishes containing FETAX solution (625 mg/L NaCl, 96 mg/L $NaHCO_3$, 30 mg/L KCl, 15 mg/L $CaCl_2$, 60 mg/L $CaSO_4 \cdot 2H_2O$, and 70 mg/L $MgSO_4$, Sigma) until they reached NF stage 8 (midblastula), according to Nieuwkoop and Faber [5].

To check if the FETAX-score can measure developmental degree variations, samples were then maintained throughout the R-FETAX period (6 days) in different thermostatically controlled conditions, with temperatures set at 17°-20°- 23° or 26°C. These temperatures are within the physiological range, and are considered able to change developmental speed without inducing abnormalities [16].

Groups exposed to Eth (Fluka, purity ≥ 99.5 %) were maintained at standard 23°C temperature throughout the entire test period (6 days). During the first two experimental days (morphogenetic period, sensitive for teratogenicity) embryos were statically exposed (without medium renewal) to Eth directly dissolved in FETAX to obtain Eth concentrations 0–0.1 % (concentration used when Eth is used as a solvent), 0.25 %, 0.5–1–1.5–2 % v/v (similar to those reported in the literature [17–23]).

At the end of the test (day 6), living tadpoles were overdosed with an anaesthetic (MS222, Sigma) (0.5 % dissolved in FETAX solution). Euthanized tadpoles were rinsed in FETAX, fixed in 50 %ethanol and preserved in 70 % ethanol. Tadpoles were morphologically evaluated under a dissecting microscope (Leica), scored and photographed. Any abnormalities were also recorded.

2.3. Mathematical modelling (PROAST)

The software package PROAST (70.3 version) developed by the Dutch National Institute for Public Health and the Environment (RIVM) (www. proast.nl) for the statistical analysis of dose-response toxicological data, was used for modelling. The benchmark dose approach was applied to the datasets, deriving the Critical Effect Dose (CED) at a Critical Effect Size (CES) set at −0.05 percent change in the mean response compared to the mean response in the controls ($CED_{-0.05}$, used as point of departure in modern risk assessment).

2.4. Statistical analysis

Quantal data were analysed by Chi-square for trend, and quantitative data were analysed using ANOVA followed by Tukey's test. The level of significance was set at $p < 0.05$.

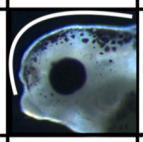
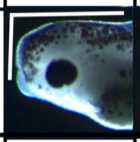
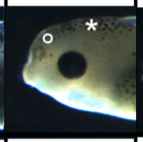
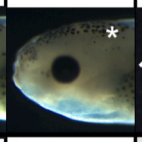
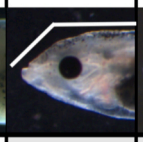
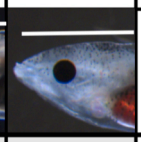
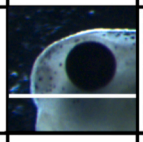
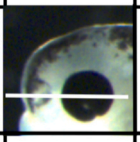
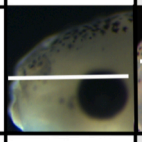
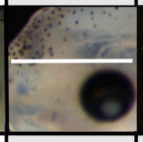
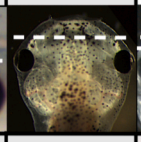
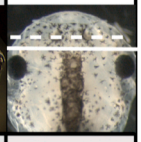
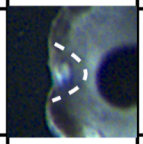
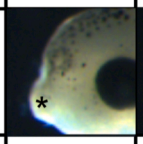
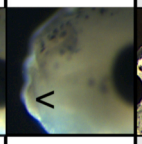
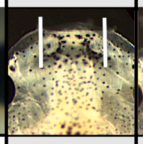
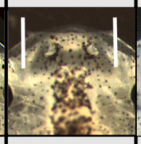
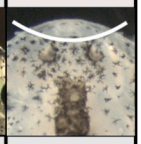
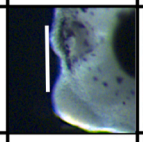
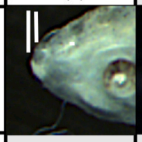
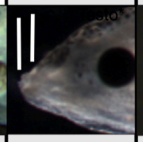
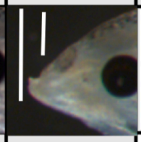
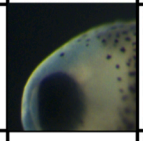
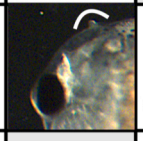
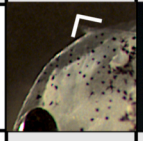
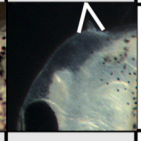
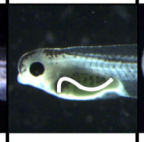
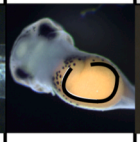
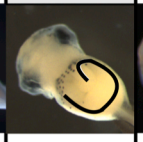
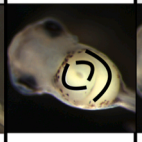
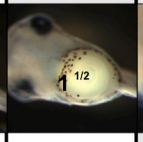
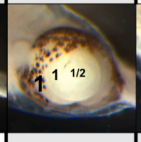
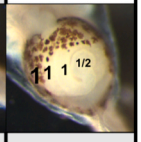
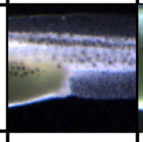
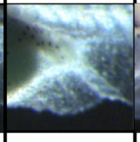
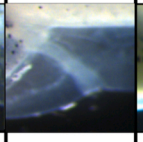
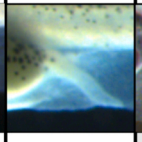
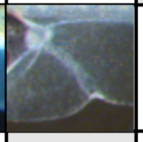
3. Results

3.1. FETAX-score parameters

The developmental score sheet was created by considering the normal morphology of NF stages 40–47 (according to Nieuwkoop and Faber [5] and web resource [12]) focusing on seven easily recognizable characteristic features: head, naris, mouth, lower jaw, tentacles, intestine, anus (Fig. 1 showing graphical annotations on pictures; for the native pictures without annotations refer to the figure in supplemental material). Total scores, theoretically achievable at each stage, were calculated as the sum of individual scores (in the absence of stage-specific descriptions we referred to the previous score level: i.e. at stage 41 the head score is 0).

3.2. Temperature-dependent FETAX-score data

At the end of the test period (day 6), after evaluation under a dissecting microscope (Leika), tadpoles developed at different temperatures (17–20- 23–26°C) were morphologically examined and scored; individual total scores were then calculated. As expected in ectothermic animals like amphibians, the total scores reflect the dependency of *Xenopus* development on temperature (Table 1; Fig. 2). By contrast, the temperature range used did not induce abnormalities, suggesting that

NF stage	40	41	42	43	44	45	46	47
SCORE	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**
		Similar to stage 40 SCORE= 0						Similar to stage 46 SCORE= 6
Head	Rounded	---	Squared	Emispheres (°) hindbrain(*) enlarged	Hindbrain stretched (*)	Trapezoid	Linear brain vesicles	---
		Similar to stage 40 SCORE= 0		Similar to stage 42 SCORE= 2				
Naris (inferior border)	Aligned with eye inferior border	---	Aligned with eye pupil	---	Aligned with eye superior border	Above the eye superior border	Dorsal (close to forebrain)	Rostral (far from forebrain)
		Similar to stage 40 SCORE= 0	Similar to stage 40 SCORE= 0					
Mouth	Circular	---	---	Flat, adhesive organ (*) trapezoid	Flat, adhesive organ linear (<)	Oval, alligned with naris border	Oval beyond the naris	Cup-shaped
		Similar to stage 40 SCORE= 0	Similar to stage 40 SCORE= 0	Similar to stage 40 SCORE= 0				Similar to stage 46 SCORE= 6
Lower jaw	Aligned with maxilla	---	---	---	Slightly anteriorly protruding	Anteriorly protruding	Markedly anteriorly protruding	---
		Similar to stage 40 SCORE= 0	Similar to stage 40 SCORE= 0		Similar to stage 43 SCORE= 3			Similar to stage 46 SCORE= 6
Tentacles	Absent	---	---	Bud	---	Conical	Elongated	---
								
Intestine	I- shaped	S- shaped	C-shaped	G- shaped	Coiling	Spiralized (1 ½ revolutions)	Spiralized (2-2 ½ revolutions)	Spiralized (3-3 ½ revolutions)
							Similar to stage 45 SCORE= 5	Similar to stage 45 SCORE= 5
Anus	90° with tail	60° with tail, conical	Turned, L-shaped	Hoof-shaped	Hoof-shaped, narrow	45° with tail	---	---
TOTAL SCORE	0	3	8	18	27	35	41	45

(caption on next page)

Fig. 1. FETAX-score sheet describing the characteristics of seven features during development, according to NF stages 40–47. **Head**: starting from a round head, cranium development progressively aligns brain vesicles, passing through squared and trapezoid morphologies. During this process, brain vesicles initially enlarge, then stretch. **Naris**: due to cranio-facial development, the naris position, initially aligned with the eye's inferior border, gradually becomes dorsal. Initially the naris are closed to the olfactory forebrain bulbs, then they move the a rostral position as the naris appear far from the forebrain, and the olfactory nerves become visible. **Mouth**: when the stomodaeum opens,the mouth appears as a perfect circle on the top of the developing adhesive organ. During facial morphogenesis, the mouth becomes a flat opening and the adhesive organ gradually regresses. Finally, the mouth appears as the most anterior structure, with an oval to cup-shaped morphology. **Lower jaw**: an index of facial development, the lower jaw, initially aligned with the maxilla, progressively reaches a macrognathic position, protruding anteriorly. **Tentacles**: sensory organs formed on the upper lip border, they develop as a bud (round) and elongate, passing through a conical shape. **Intestine**: previously considered the simplest and most indicative parameter to detect *Xenopus* tadpole developmental degree, the initial linear gut shape progressively lengthens and reaches S- C and- G shapes, finally, coils into a spiralized structure. **Anus**: originating from the blastopore, the antero-verted anal canal characterizes embryonic stages, and in NF 40 tadpoles, it is perfectly at 90° with the tail. In later stages, the anal canal gradually becomes retro-verted and stretched, assuming a transitory hoof-shaped structure with an enlarged anal opening. To use the score sheet, each of the seven features is examined and scored, and the total of assigned scores for each parameter is the total score. Total scores, theoretically reachable at each stage, were calculated as the sum of individual scores. In the absence of stage-specific descriptions we referred to the previous score level: i.e. at stage 41 the head score was 0. The most common scores assigned in our *X. laevis* tadpoles developed at 23°C are shown in grey. NF= Nieuwkoop and Faber stages, according to Nieuwkoop and Faber (1956) and the web resource (http://www.xenbase.org/, RRID:SCR_003280).

Table 1
Temperature-related increase of total score (M±SD) in samples maintained in thermostatically controlled conditions (17–20- 23–26°C) for the whole test period (6 days). 23°C is the standard maintenance temperature in FETAX tests. Tukey's Post-hoc Test: [a] $p<0.05$ vs T17; [aa] $p<0.01$ vs T17; [bb] $p<0.01$ vs T20; [cc] $p<0.01$ vs T23.

	17°C N=15	**20°C** N=15	**23°C** N=15	**26°C** N=15
			aabb	aabb
Head	1.87±1.60	2.60±1.30	5.07±0.59	5.80±0.41
		a	aabb	aabbcc
Naris	2.53±0.74	3.20±0.77	4.00±0.00	5.53±0.74
		aa	aabb	aabb
Mouth	0.93±1.67	2.80±1.86	5.33±0.49	5.93±0.46
			aabb	aabbcc
Lower jaw	0.00±0.00	0.53±1.41	4.20±0.41	5.73±0.70
			aabb	aabbcc
Tentacles	0.40±1.06	1.20±1.52	3.67±0.98	5.00±0.00
			aa	aabbcc
Intestine	4.33±1.05	5.07±0.80	5.47±0.64	6.47±0.64
			aa	aabbcc
Anus	2.67±0.82	3.07±0.46	3.53±0.52	4.93±0.70
		aa	aabb	aabbcc
Total score	**12.73±4.23**	**18.47±5.29**	**31.27±2.25**	**39.40±2.50**

the proposed scoring system is sensitive enough to detect effects (young/old-for-age phenotypes) at sub-teratogenic conditions. Notably, the total score obtained in tadpoles maintained at 23°C (the temperature indicated by the Nieuwkoop and Faber staging paper [5]) was 33.27 ± 2.25. This value does not correspond to the theorical NF stage 46 full score (41, as shown in Fig. 1), suggesting different developmental conditions in our laboratory compared to 1956 Nieuwkoop and Faber staging (possibly related to differences in strain, mating procedures, maintenance equipment, etc).

3.3. Effects of ethanol on FETAX-score

To evaluate the applicability of FETAX-score in detecting minor xenobiotic-related alterations in developmental timing, *X. laevis* embryos exposed to non- teratogenic Eth concentrations were scored. As all tadpoles exposed to 2 % v/v Eth resulted malformed (22 % plurimalformed tadpoles, 78 % tadpoles showing oedema, hydropericardium, head defects, ocular malformations) (Table 2) this group was excluded from scoring. Tadpoles exposed to 1.5 % v/v Eth showed some morphological aspects (head/mouth shape) (Table 2) attributable to developmental delays, therefore this group was included in scoring procedures along with the other groups (Eth 0–0.1–0.25–0.5–1 % v/v,

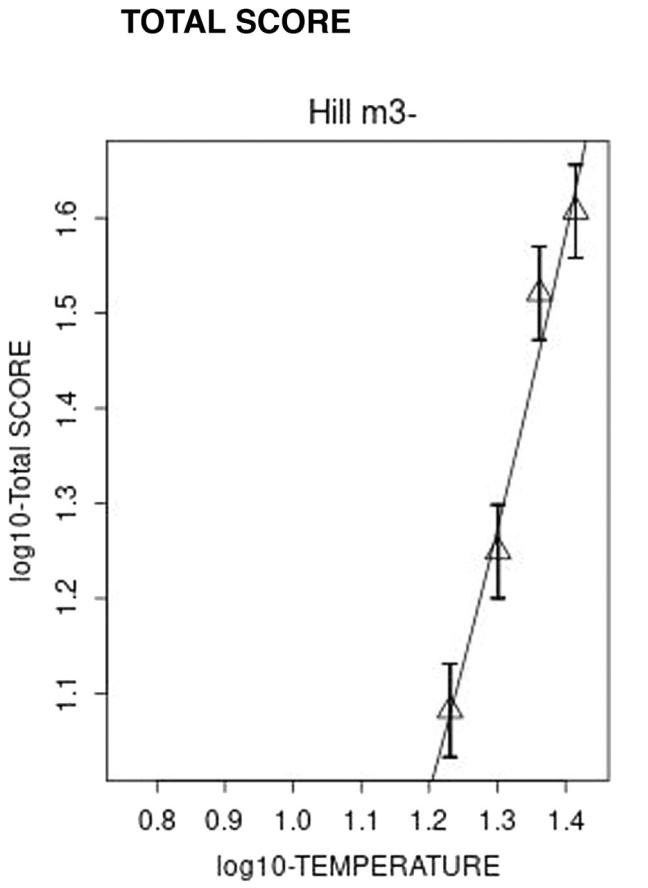


Fig. 2. Temperature-related total FETAX-score, indicative of the general developmental degree, obtained in groups maintained at 17–20- 23–26°C for the whole test period (6 days) and modelled by PROAST software package (exponential models applied).

where no abnormalities were recorded). Based of Table 2 data, the no adverse effect level (NOAEL, classically used as a point of departure in risk assessment) was determined to be 1 % Eth.

By contrast, decreases in FETAX-scores for the seven different single parameters and the total score were also observed in the 1 % v/v Eth group (Table 3), highlighting the need to reposition the NOAEL at 0.5 %. Modelling (PROAST approach) showed a clear dose-relationship in all

Table 2
Morphological characteristics of tadpoles in groups exposed to Eth 0–2 % v/v. Statistics (Chi-square for trend) on frequencies.

	Eth 0 % (N=15)	Eth 0.10 % (N=15)	Eth 0.25 % (N=15)	Eth 0.50 % (N=13)	Eth 1 % (N=14)	Eth 1.50 % (N=13)	Eth 2 % (N=9)	p (Chi-square for trend)
Malformed tadpoles (%)	**0**	**0**	**0**	**0**	**0**	**0**	**100**	**0.0000001**
Plurimalformed	-	-	-	-	-	-	22	
Head defects	-	-	-	-	-	-	22	
Eye malformations	-	-	-	-	-	-	22	
Hydropericardium	-	-	-	-	-	-	67	
Ventral oedema	-	-	-	-	-	-	11	
Tadpoles with developmental delays (%)	**0**	**0**	**0**	**0**	**0**	**38**	**—**	**0.00016**
Head: not linear encephalon with micrognathia	-	-	-	-	-	*38*		

Table 3
FETAX-scores (M±SD) in groups exposed to Eth 0- 1.5% v/v. Tukey's Post-hoc Test: [a] $p<0.05$ vs Eth 0 %; [aa] $p<0.01$ vs Eth 0 %; [bb] $p<0.01$ vs Eth 0.1 %; [c] $p<0.05$ vs Eth 0.25 %; [cc] $p<0.01$ vs Eth 0.25 %; [dd] $p<0.01$ vs Eth 0.5 %; [ee] $p<0.01$ vs Eth 1 %.

	Eth 0 % N= 15	**Eth 0.1 %** N= 15	**Eth 0.25 %** N= 15	**Eth 0.5 %** N= 13	**Eth 1 %** N= 14	**Eth 1.5 %** N= 13
Head	5.47±0.83	5.87±0.35	5.40±0.91	5.54±0.78	5.57±0.65	a bb c dd ee 4.46±0.97
Naris	6.80±0.41	6.27±0.46	6.33±0.49	6.00±0.41	6.64±1.08 a	aa bb cc dd ee 3.08±1.50
Mouth	6.00±0.00	5.47±0.52	5.47±0.52	5.62±0.51	4.79±1.42 aa bb cc dd	aa bb cc dd 4.08±1.85
Lower jaw	4.60±0.51	4.47±0.52	4.47±0.52	4.46±0.52	2.86±1.88 aa bb cc dd	aa bb cc dd ee 1.23±1.92
Tentacles	3.00±0.00	3.00±0.00	3.13±0.52	3.46±0.88	0.86±1.41 a bb c	aa bb cc dd 0.46±1.13 c
Intestine	5.47±1.06	5.80±0.86	5.87±0.35	5.85±0.38	4.64±1.08	4.85±1.34
Anus	3.87±0.83	3.27±0.59	3.33±0.49	3.23±0.44	3.93±0.62 aa bb cc dd	3.08±0.28 aa bb cc dd ee
Total score	**35.20±2.34**	**34.13±1.68**	**34.00±1.31**	**34.15±1.95**	**29.29±5.36**	**21.23±3.98**

FETAX-score parameters except for the anus and identified, based on the derived Benchmark Doses (CEDs, setting the critical response at CES-$_{0.05}$), tentacles as the most sensitive structures (Fig. 3, Table 4).

4. Discussion

In addition to lethality and teratogenicity, the quantitative estimation of embryonic growth is a major concern in developmental toxicology evaluation. The present work aimed to identify and test a scoring method to evaluate young/old-for-age phenotypes in *X. laevis* tadpoles at the end of FETAX procedures. The method was based on Nieuwkoop and Faber staging [5,12] and assessed in samples developed at different maintenance temperatures. The obtained results suggest that the FETAX-score is sensitive to moderate distress not influencing general morphology (sub-teratogenic conditions). To test the applicability of the FETAX-score in detecting xenobiotic-related developmental delays, data on *X. laevis* embryos exposed below teratogenic concentrations of Eth (0–0.1- 0.25–0.5- 1–1.5 %) were assayed. Results on the FETAX-score support the specific activity of Eth in inducing developmental delays (young-for-age phenotype) at sub-teratogenic concentrations, showing tentacles as the most Eth sensitive embryonic target.

Literature on *X. laevis* development describes, after continuous exposure to Eth 1–2–2.5 % v/v from late blastula stages until the end of the test, a typical phenotype (microcephalic, microphthalmic,or phenotypes with multiple malformations including ventral oedema) [17–22]. In the present work, the exposure was limited to the first two days of development (NF 10–40, morphogenetic period) and marked teratogenic effects were obtained only at the highest concentration (Eth 2 % v/v). In contrast, at lower (not teratogenic) concentrations, the dose-dependent decrease of total- and single-parameter scores (except anus) highlighted the presence of an Eth-related young-for-age phenotype. In humans, the alcohol-related spectrum of physical, cognitive, and behavioral disabilities in newborns is known as fetal alcohol spectrum disorder (FASD) [24–26]. The most severe form, which includes morphological abnormalities, is defined as fetal alcohol syndrome (FAS) [27–29].

Interestingly, facial features and growth deficiency are the most distinctive human FASD morphological characteristics [30]. Notably, according to Astley and Clarren [31], the four key diagnostic features of FAS are, in order: 1) growth deficiency, 2) FAS facial phenotype, 3) brain dysfunction, 4) gestational alcohol exposure; growth deficiencies and the FAS facial phenotype, in particular, are highly correlated with, and predictive of, severe brain dysfunctions (in detail: small palpebral fissures, a smooth philtrum and a thin upper lip linearly correlate, among individuals with prenatal alcohol exposure, to cognitive impairment) [31,32]. Our scoring method allowed us to describe a dose-dependent general developmental delay (total score decrease) at not teratogenic concentration levels, highlighting tentacles (expansions of the upper lip, a key structure in FASD) as the most sensitive structure, affected at Eth concentrations unable to induce gross abnormalities.

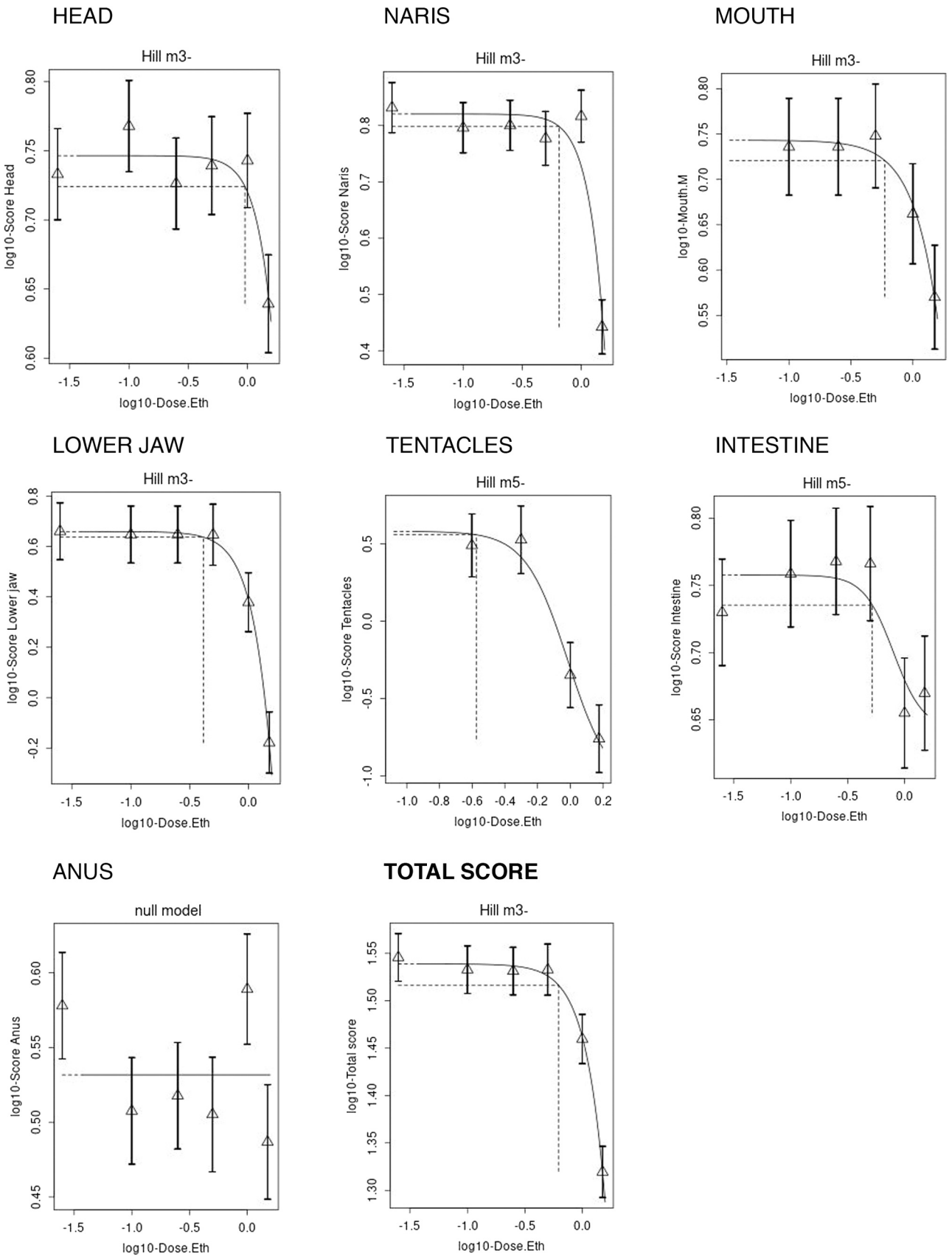


Fig. 3. Concentration-dependent reduction of single FETAX-scores and total FETAX-score in *X. laevis* tadpoles exposed to Eth (0–1.5 % v/v) during the organogenetic stages (PROAST modelling). Tentacles were the most sensitive parameter.

Table 4
Benchmark Doses derived for each parameter setting CES at −0–05 (CED-0.05) with their Confidence Intervals (CEDL-CEDU).

	$CED_{-0.05}$	CEDL	CEDU
Head	0.95	0.74	1.07
Naris	0.65	0.60	0.68
Mouth	0.59	0.17	0.89
Lower jaw	0.41	0.25	0.52
Tentacles	0.27	0.10	0.32
Intestine	0.52	0.36	0.74
Anus	_	_	_
Total score	0.62	0.46	0.77

In conclusion, the overall obtained results support the use of this innovative scoring system to quantitatively assess alterations in the rate of development, useful, in embryotoxicity studies, to detect sub-teratogenic effects.

Statements and Declarations

NA

Ethical approval

The study was conducted according to the relevant European (EU Directive 2010/63/EU for animal experiments) and Italian (Legislative Decree No. 26/2014) laws, rules, and regulations. All procedures were examined and approved by the Animal Welfare Organization of the Università degli Studi di Milano. Facility authorization number: 198283; date: 19/12/2019.

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Authors'contribution

All authors contributed to the study conception and design. Material preparation was performed by M. Battistoni, F. Di Renzo, R. Bacchetta, E. Menegola; data collection and analysis were performed by M. Battistoni, F. Di Renzo, E. Menegola. Data modelling was performed by F. Metruccio, E. Menegola. The first draft of the manuscript was written by E. Menegola; all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests. Elena Menegola reports financial support was provided by University of Milan. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

Data availability

Data will be made available on request.

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