

## Research Article

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## Four-Eyed Fish (*A. anableps*) Fingerlings Demonstrated Multiple Birth Defects Suggestive of Nutritional Deficiencies

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### ABSTRACT

In a colony of four-eyed fishes (*A. anableps*) some females produced offspring demonstrating birth-defects. At external inspection, the ventral midline was open over variable lengths. In some cases, it was combined with lordosis, kyphosis, or scoliosis. Affected specimens also suffered from cataracts. Histopathology revealed widely scattered degeneration of myofibers. Sheath cells of the notochord had proliferated, resulting in asymmetric shape distortion of vertebrae and indentations in the spinal cord. The spinal cord revealed an extensive axonal degeneration.

The liver appeared mottled due to focal fat infiltration. Cataracts of ocular lenses appeared as vacuolization of lens fibers. The multiple pathologies noted may have been due to nutritional imbalances because the colony received a carnivore-based diet. Feeding ecology of *Anableps* is one of omnivory with a predominance of herbivory. This may have resulted in lesions comparable to deficiencies in Vitamin E, Vitamin C, and Selenium.

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**Received:** January 31, 2024; **Accepted:** February 06, 2024; **Published:** February 13, 2024

**Keywords:** Four-Eyed Fish, *A. anableps*, Histopathology, Deficiency, Vitamin E, Selenium, Vitamin C, Cataract, Omnivory

### Introduction

*A. anableps*, the largescale 'four-eyed' fish is an elongate fish found mainly in freshwater, and occasionally in brackish waters of lagoons, river deltas, and coastlines. *A. anableps* prefers a biotope of Rhizophora mangrove forests. It inhabits shallow margins of permanent water channels during low tide moving for foraging into inundated zones during high tide [1].

*A. anableps* has an omnivorous feeding habit with a predominance of plant material. In wild-caught specimens, red algae (*Catenella* spp.) fill 80% of the stomach contents. Further food items consist of diatoms (microalgae) living on shore mud, insects, other invertebrates, and small fishes [2]. Overall mean daily consumption is 9.6% of body mass. The quantity of food consumed is influenced by tide, time of the day, and site. Main meal is in the morning [1]. Females can grow to a length of  $\pm 18$  cm with a maximum of 51 cm. Males reach a length of  $\pm 14$  cm [3].

Embryonal development in the mother lasts approximately two months. At birth, the skeleton is fully developed, inclusive of vertebrae and ribs. Mean fecundity is 12 eggs/embryos per female (range: 1-37 eggs/embryos) [4].

*Anableps* is a small genus. It comprises 1) *A. anableps* from northern South America and Trinidad. 2) *A. microlepis* living in

middle and south America. 3) *A. dowei*, endemic in the Pacific area from Nicaragua to Mexico [5].

Four-eyed fishes swim in the waterline. The water meniscus bisects the eyes. Eyes are raised above the top of the head. The frontal bone is expanded to accommodate eyes [6]. Lenses are split in two by a horizontal band of tissue. Each half has its own pupil and retina [7]. It allows animals to see under and above the surface of the water at the same time. Development of upper and lower parts of eye is regulated by unique photoreceptor genes [8]. Cornea and pupils are duplicated already in a larval stage [6].

This paper describes macroscopic and more especially microscopic lesions suggestive of multiple deficiencies in *A. anableps* for the first time. Suggestions are given to prevent future cases.

### Materials and Methods

Materials for this study included fingerlings from a zoo-housed colony of four-eyed fish (*A. anableps*). Population in the aquarium consisted of:

- A cohort of imported adult (normal) specimens.
- Specimens born in captivity, and grown to adulthood while showing postural deformations.
- A mixture of normal fingerlings and individuals of either sex, with a manifest delayed closure of the celom. Some of these also showed vertebral abnormalities caudally to the dorsal fin.

At the start, the colony comprised 10 animals of either sex. Four years later 12 wild-caught specimens (sex not determined) were added, and three years later a cohort of seven specimens. Animals were housed in a large aquarium at 24 – 28 °C. Adult fishes were fed twice daily.

In the morning they received Tetraphyll® (Tetra GmbH, Herrenteich 78 Melle, 49324 Germany). Equal portions were given of water fleas (*Daphnia pulex*) mosquito larvae (*Culex sp.*) (either fresh or deep-frozen) mysid shrimps (*Mysis spp.*) krill (mixed species) pieces of North Sea grey shrimps (*C. grangon*) bream (*Abramis brama*) (pieces) and, beef (*Bos taurus*) (heart, pieces) [4].

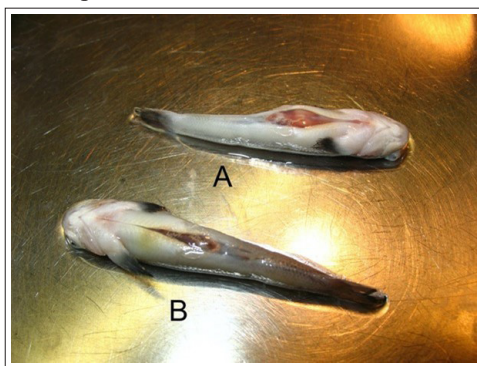
During the period the colony was maintained, fifty fingerlings from (most probably) unrelated *A. anableps* parents, were found dead. Twenty-one demonstrating gross pathologies were submitted for post-mortem examination. Two adult specimens born in this aquarium under identical circumstances, suffering from either kyphosis or scoliosis, were submitted as well. Radiographs were taken from malformations of the spinal column in these 2 adult males.

Samples were fixed in 4% neutral buffered formaldehyde and embedded in paraffin. Slides, 5 µm in thickness were stained with hematoxylin and eosin.

## Results

### Macroscopy

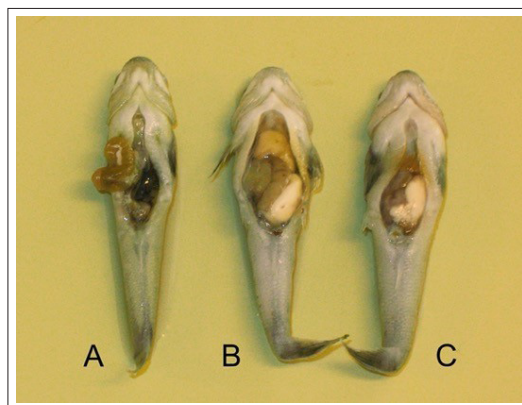
Abdominal midline defect in fingerlings was a slit in the celomic wall. Slits were perfectly symmetrical and of elliptical form with smooth linings. Minor alterations showed a tapering body combined with a narrow slit located in the middle of the celomic cavity (Figure 1A). In more pronounced cases the body showed a bulging celoma (Figure 1B) with a slit reaching from a point between branches of the mandible extending to immediately cranially of the genital porus. Celomic organs were located within the celomic cavity held together by a thin transparent membranous envelope, the trophoderm.



**Figure 1:** *A. anableps*. The Midline Closure is Delayed and Variable. One Specimen Shows a Bulging Abdomen and a Wide Slit (A). In the Other one (B) the Closure is Slightly more Progressed. This body is Tapering and the Slit is Narrow. The Organs are Enclosed in the Trophoderm.

Skeletal deformations concerned the vertebral column caudal of the anal fin. Malformations seen were lordosis kyphosis and scoliosis. In some cases, it was already manifest at the moment of birth (Figure 2). Changes varied from only a few vertebrae to some 5 or 12. Some misshaped juveniles had under prevailing circumstances grown to adulthood. Deformation of the vertebral

column was located immediately caudal of the dorsal fin (Figure 3). These animals were normal in their attitude.



**Figure 2:** *A. anableps*. Delayed Midline Closure Combined with Vertebral Scoliosis to the left and the Right (B, C). In Specimen (A), the Trophoderm is Ruptured.

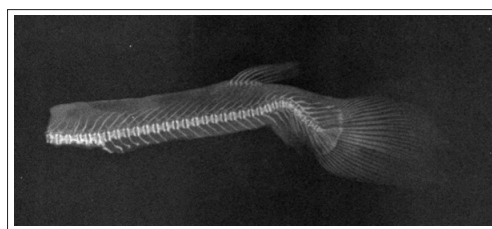


**Figure 3:** *A. anableps* (1-0). Localized Kyphosis was Distal to the Dorsal Fin.

At inspection, eyes of fingerlings were macroscopically normal.

### Radiography

In full-grown-animals radiography of affected area of the spinal cord showed a marked localized deformation of a coherent series of vertebrae. Vertebrae on both ends of the curvature appeared normal (Figure 4).



**Figure 4:** *A. anableps* (1-0) Radiograph of the Same Specimen as in Fig. 4, Showing Distal to the Dorsal Fin Localized Kyphosis and Misshaped Vertebrae.

### Microscopy

Rod-shaped bacteria were seen loosely seeded over the parietal peritoneum. Signs of invasion of the bacteria in the tissues were not found. The serosa of celomic organs, such as the intestine located in the trophoderm was free of organisms.

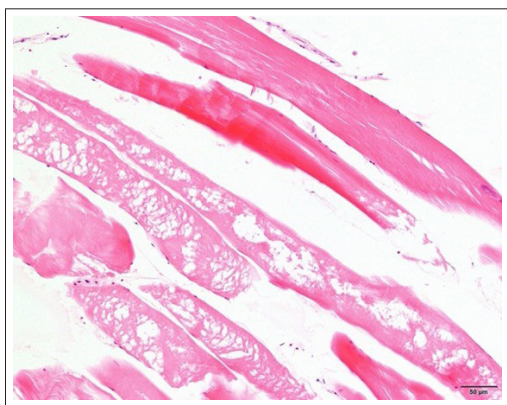
Parietal peritoneum was covered with a mesothelium followed by a fascia. Between mesothelium and fascia, melanophores were situated in variable density. At the external surface of the fascia, a layer of thin muscle fibers was present. These demonstrated a distinct cross banding. Fibers were haphazardly arranged and separated by an optically empty area. Scattered between small fibers some fibers of a thickness identical to muscle fibers of deeper muscles were present. Thick fibers contained multiple nuclei



directly below the sarcolemma. Especially in cross-sections, it was seen that in the last-mentioned cells myofibrils were loosely arranged. Degeneration of newly produced muscle fibers was not observed.

Muscles of trunk and head showed degeneration of myofibers. Fibers were not always uniformly affected. Certain areas were but slightly and diffusely involved while in others 100 percent of fibers have degenerated. Outlines of 100 percent degenerated areas were unsharp. In the marginal zone degenerated myofibers mixed with normal ones. Some of the affected myofibers were swollen. Only part of them revealed birefringence.

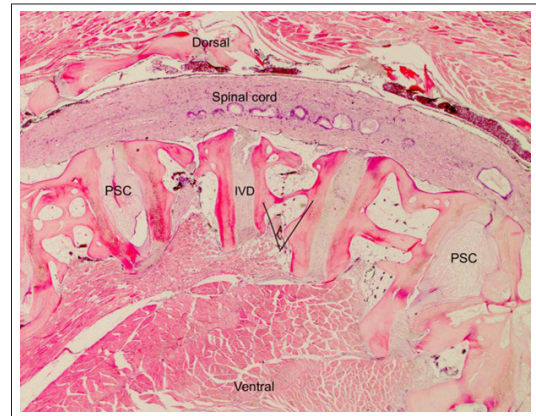
More centrally in an area of degeneration, myofibers revealed a variety of changes. Within a myofiber, eosinophilic material could be present either as crossbands, as irregularly distributed clusters, or as accumulation along the perimysium (Figure 5). This eosinophilic material was recognized as clusters of myofibrils. Especially in crossbands, margins were irregular with myofibrils extending in the optically empty surroundings. It made an impression as being torn apart by forces of degenerating myofibrils. Optically empty areas were free of birefringence. Mineralization was not observed. There was some difference between individuals. In a second specimen, some hyalinization was present in the eosinophilic areas. In hyalinized areas, myofibrils were not recognizable. These areas were non-birefringent. Inflammatory cells were absent.



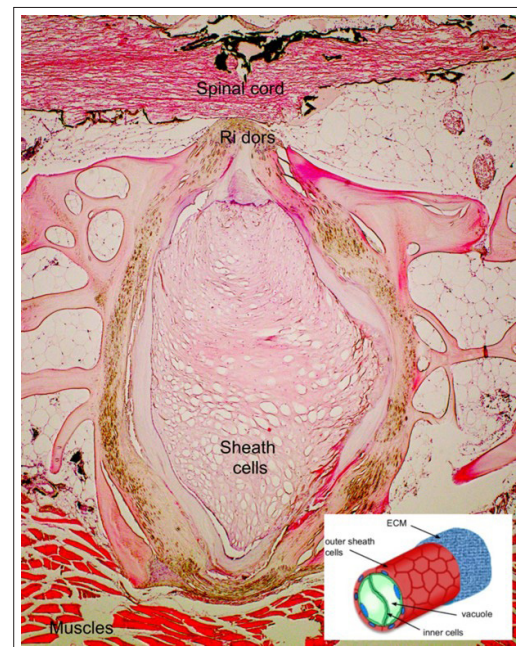
**Figure 5:** *A. anableps*. Muscular Fibers Degenerated. The Sarcoplasm is Clumped Together in Irregular Masses.

Vertebrae in the curvature were misshaped. In the case showing kyphosis, vertebrate bodies were vertically wedge-shaped (Figure 6). The larger side was situated along the spinal cord. In case of lordosis, deformities were accordingly. Arrangement of bone spicules was different in every vertebra. In some vertebrae, a single major spicule extended like a bridge between the cranial and the caudal wall of vertebrae. In others, spicules produced a network. Intervertebral discs (IVD) in the area of curvature showed some variation. A few discs were more or less quadrangular slender structures. In these the normal anatomy was present. A cartilaginous endplate (CEP) covering vertebral bone was 2-3 cell-layer in thickness. It was covered by a layer of parallel arranged connective tissue fibers showing long slender nuclei. Both dorsally and ventrally, fibers made a curve to level dorsal and ventral surfaces of vertebrae. The nucleus pulposus (NP) was an unstructured mass. It was present as a retracted sharp delineated homogenous acellular eosinophilic mass in the center of the intervertebral disc.

Most of IVDs present in the tissue slides were markedly changed (Figure 6). In altered IVDs the CEP had disappeared. A layer of hyalinized material filled the gap. Dorsal and ventral parts consisted of connective tissue fibers which were compressed to produce a ridge. Ridges protruded in a dorsal and ventral direction. Dorsal protrusions impinged on the spinal cord making minor indentations in the spinal cord. Ventral protrusions extended between muscles (Figure 7).



**Figure 6:** *A. anableps*. The Curvature Shows Wedge-Shaped Vertebral Bodies (V-lines). The Normal Intervertebral Disc (IVD) is Quadrangular and Slender. Altered IVDs show a larger Space, Filled with Proliferated Sheath Cells (PSC). There is Some Indentation of the Spinal Cord.



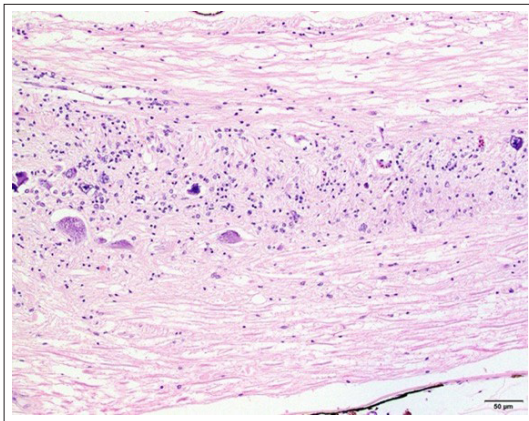
**Figure 7:** *A. anableps*. The dorsal and Ventral parts of the IVD are Compressed, Producing Protruding Ridges. The Dorsal Protrusion Impinges on the Spinal Cord, Making a Minor Indentation (Ri dors). The Ventral Protrusion (Ri vent) Extends Between Muscles.

The Widened Center of the IVD's is Filled with a Mass of Cells Resembling Notochordal Sheath Cells (Sheath cells). Inset: Drawing of the Notochord (Courtesy M. Bagnat).



These IVDs were broadened and filled with masses existing of large closely arranged, angular cells with a firm outer membrane and pyknotic nuclei. Protoplasm was homogenous. There was no recognizable intercellular material. They most probably had proliferated and produced well-delineated homogenous masses. These cells resembled sheath cells of the notochord. In other IVD's smaller areas of a homogenous eosinophilic substance most probable edema was present (Figure 6). Locally, bundles of birefringent connective tissue entered the masses. In a few cases, there were heavy strands of connective tissue dividing the mass into compartments of varying sizes.

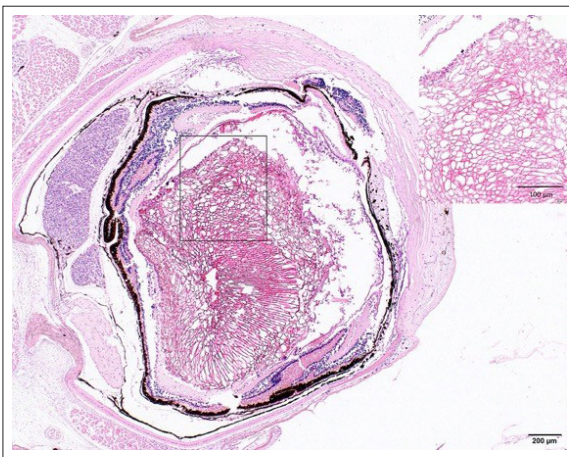
**The spinal cord:** Around the central canal of the spinal cord tissue and ganglion cells appeared normal. The outer areas, both dorsally and ventrally, revealed extensive axonal degeneration. It consisted of loosely longitudinally arranged fibers (Figure 8).



**Figure 8:** *A. anableps*. Spinal Cord. Extensive Axonal Degeneration. HE Obj. 10.

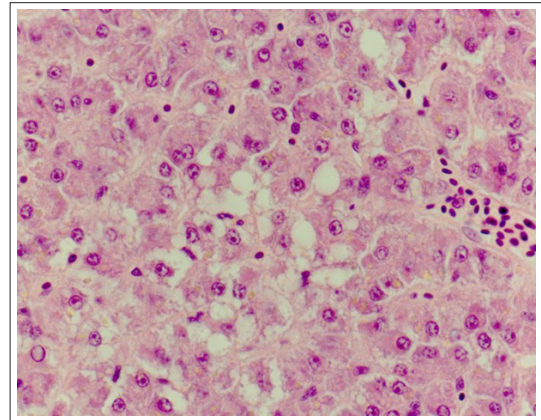
Dorsal root ganglia located just lateral to each segment of the spinal cord appeared normal.

**The Eyes:** Lenses of the eyes developed vesicles in the fibers. It started centrally in the innermost fibrous area as small vesicles. Towards the periphery, vesicles gradually enlarged. Larger vesicles had a rather thick wall. They then disintegrated to produce, in connection with neighboring vesicles in the same stage, an optically empty zone surrounded by the lens capsule (Figure 9).



**Figure 9:** *A. anableps*. Eye. Cataract. In the lens, the Fibers are Progressively Swollen and Vacuolated. HE Obj. 4. Inset: Detail. Lens Fibers are Swollen, Vacuolated, and Degenerated. HE Obj. 10.

**The Liver:** The liver showed a faintly mottled aspect caused by multifocal areas of cells showing intracellularly optically empty vesicles or areas (Figure 10). Vesicles appeared as vacuoles of fat dissolved during tissue processing.

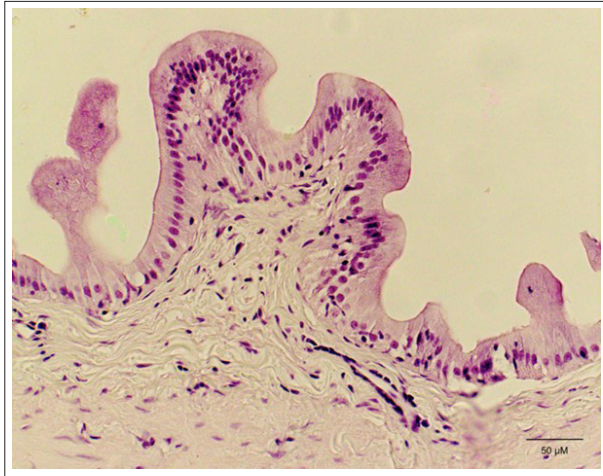


**Figure 10:** *A. anableps*. Liver. Area of Hepatocytes Loaded with Fat. HE Obj. 25.

### Discussion

Knowledge of embryonal development is a help in understanding development of birth defects. *Anableps* species are ovoviviparous. Egg cells are fertilized while in follicles in the ovary. Embryos develop in modified ovarian follicles within the abdominal cavity of the female until parturition. Egg cells are small and contain little yolk. A small yolk mass is absorbed early in development [9]. Development of embryos is highly matrotrophic. Embryos absorb maternal nutrients by using extra produced "trophoteniæ". These are specialized extensions of the hindgut. They are surrounded by fluids embedding the developing embryo. Visceral organs of the embryo protrude into an extra-embryonic pericardial sac called "trophoderm". It is indicated as an exteriorization of celomic organs. Embryonic weight-increase results from nutrient transfer across a "follicular placenta".

This complex structure is produced by apposition of maternal follicular epithelium to absorptive surface cells of the embryo's pericardial trophoderm (Figure 11) [3]. The external surface of the pericardial trophoderm develops hemispherical projections termed vascular bulbs. Within each bulb, a vascular plexus of the trophoderm expands to form a blood sinus. The hindgut of the fetus expands and is almost filled with vascularized villi. This expanded region is believed to absorb and digest follicular fluid [3,9]. Increase of embryonic weight results from nutrient transfer across these structures. Postfertilization weight increase is  $\pm 298.000\%$  [3]. Closure of the abdominal midline occurs in the very last period of development [10]. It is an intriguing unsolved question how *Anableps* species modified their morphology, physiology, and gene expression profile to adapt to this reproductive strategy.



**Figure 11:** *A. anableps*. The Pericardial Trophoderm is Characterized by Hemispherical Projections. He obj. 25.

Central point in this discussion is the importance of Selenium (Se), Vitamin E, and Vitamin C. All three are important to protect tissues against deleterious effects of H<sub>2</sub>O<sub>2</sub> and organic (lipid) hydroperoxides.

Selenium has been identified as an essential micronutrient in all vertebrates. It is required at the most fundamental physiological level [11]. Biological effects of selenium are largely mediated by selenium-containing proteins (selenoproteins). Se in combination with glutathione peroxidase destroys lipid hydroperoxides [12].

Vitamin E prevents formation of lipid hydroperoxides and protects against oxidant damage to membranes [13].

Vitamin C is an oxygen scavenger that reacts with oxygen and removes it. It prevents damaging effects of oxygen and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). Vitamin C has dual roles. It has antioxidant properties and is involved in synthesis of collagen. Severe deficiency in vitamin C leads to cataract [14].

Nutritional myopathy, caused by deficiency in Vitamin E/Selenium has been diagnosed in a larger number of animal species. It is known from cattle swine, and sheep (lambs especially [15]. Similar cases have been reported from iguanas (*I. iguana*) and Mexican spiny-tailed Iguana (*C. pectinate*) and satanic leaf-tailed geckos [16,17].

Development of the embryo attached to the ovary is a characteristic of the small family of Anablepidae [18]. An extensive inventory of wild-caught specimens describes that all embryos present in a given ovary were at the same stage of development [19]. Rare cases of superfetation have been mentioned [8].

There was no notice of open celoms [19]. This contrasts with results obtained in captivity by various breeders. In *Anableps* sp. ventral midline defect or incomplete closure of the ventral midline at moment of birth is a well-known problem [20]. It has also been reported in the closely related *A. microlepis* [5]. As mentioned, rod-like bacteria were randomly scattered over the parietal peritoneum of the celomic wall. Most probably bacteria were washed in after birth in a passive way from aquarium water. Anecdotally, successful breeders mention hygienic measures such as frequent water changes, transport to a clean tank, feeding with live food only, and eventually use of an antibiotic (chloramphenicol). These

measurements minimize invasion of bacteria and, support a normal closure of the celomic wall. A narrow elongate slit at birth will close completely after 4-6 days [10].

Our materials revealed an abundant presence of new muscle fibers along the inner celomic wall. This indicates a potential for future closure of the abdomen. From the restricted number of materials available, it could not be decided whether production was either normal, reduced, or defective. In Atlantic salmon (*Salmo salar*) Se deficiency resulted in retarded growth and reduced survival of young fishes. Se deficient fish showed depressed activity of glutathione-peroxidase (GSH-Px) in plasma. GSH-Px protects the organism from oxidative damage. Depressed activities of GSH-Px were influenced by dietary supplementation with L-ascorbic acid. It resulted in improved growth [21]. In our materials, a deficiency in Se and ascorbic acid during embryonic growth in the ovary may have contributed to a retarded growth. We postulate that reduced production of muscles in *A. anableps* fingerlings resulted in a retarded closure of the celomic wall.

Muscular dystrophy was extensive in skeletal muscles of *A. anableps* fingerlings under examination. In the wild, the diet of *A. anableps* is omnivorous with an emphasis on vegetables. It consists mainly of red algae (*Catenella* spp.) which make up to 80% of stomach contents. Diatoms (Microalgae) are a further vegetable nutrient. It is added by insects and other invertebrates living on shore mud. Even some small fish are taken [1].

Data were insufficient to allow a secure calculation of contents in Se, vitamin E, and C in the food of the *A. anableps* under examination. Research in cultured Atlantic salmon recorded an effect of dietary supplementation with a combination of Selenium and vitamin E. This combination was found to prevent muscular dystrophy but not with either nutrient alone [23,21]. In zebrafish, vitamin E deficiency and related deficiency in vitamin C led to degenerative myopathy [22]. In the materials in our hands, lesions were consistent with nutritional myopathy. No other causes of myopathy were seen, including protozoan and metazoan parasites. This indicates that in *A. anableps* the specific combination of deficiency in Selenium and vitamin E may have caused muscular dystrophy. Unfortunately, portions of the various feeding elements were not retained for analysis. There are no data validating the composition of the diet. Remaining animals were handed over to a different zoo.

The muscular lesions were identical with established cases of Vitamin E/Se deficiency. A final diagnosis of vitamin E/Se deficiency was made.

Skeletal deformities were most probably related to muscular dystrophy. It has been suggested that in Atlantic salmon mechanical loading generated during swimming was strong enough to inhibit a normal arrangement of collagen fibrils during growth of bone [23].

In the fingerlings, muscular dystrophy was unevenly distributed through musculature. Supposedly, these dystrophic muscular fibers were unable to exert normal contractions. This will result in a non-coordinated functioning of musculature. Abnormal forces are thus exerted on the vertebral column in its growth phase. The vertebral column will divert in either direction.

The region of the curvature in the fingerlings showed a remarkable pathology of the intervertebral discs. An in-depth description of notochord in *Anableps* was not available. In zebrafish (*Danio*



*erio*) the notochord is composed of a central population of highly vacuolated cells surrounded by a layer of epithelioid cells, encapsulated by an extracellular basement membrane [24]. These cells appear morphologically identical to those in the intervertebral disc of four-eyed fishes under examination. However, there was an impressive proliferation of cells. Cells looked normal. There were no indications of malignancy.

Intervertebral discs in the *Anableps* revealed ingrowth of connective tissue. It could not be decided whether there was an interaction between sheath cells and connective tissue.

Effects on the nervous system have been recorded to be related to dietary vitamin E deficiency. Feeding healthy common carp (*Cyprinus carpio*) with various diets deficient in vitamin E resulted in time and dose-dependent swelling and degeneration of neurons [25].

Eyes of newborn *Anableps* were macroscopically normal. At histologic examination, they revealed a distinct cataract. Several factors have to be considered to explain this phenomenon. The lens is richly endowed with a complex antioxidant system consisting of non-enzymatic and enzymatic pathways which contribute to lens transparency. Major non-enzymatic antioxidants vitamin C and E play important roles [26]. Antioxidant selenium also plays a significant role in maintaining lens fibers where it supports the activity of antioxidant [34]. These antioxidants harmonize their activities. Vitamin C for instance prevents a selenite cataract [28]. Failure in this primary antioxidant defense system leads to damage to lenticular molecules, their repair mechanisms and ultimately to cataracts [29].

A conclusive problem in this study is the cause of deficiencies diagnosed in the fingerlings of *Anableps*. As problems occurred over a longer period, there may have been variations in the composition of the food. Supposedly between years and, even between seasons [14].

There is an extensive experience in commercial fish production. In trout and other fish species, vitamin C is provided in concentrations of 150 - 250 mg/kg pellets (Omoniyi and Ovie 2018) [30]. In commercial fish production, these pellets are the only food given. In the zoo, about half of the food (afternoon meal in) existed of native animal products which are poor in vitamin D. In crickets the content of vitamin E meets daily requirement (if given as sole food). Content in selenium and vitamin C depends on origin of natural materials and thus is largely unpredictable [31,32].

According to the anamnesis, (adult - pregnant) fishes in this study received their main meal in the morning. It consisted of "Sera vital insect meal®". Product description states a nutrient composition: "Vit. D3 1.800 IU/kg, Vit. E (D, L- $\alpha$ -tocopheryl acetate) 120 mg/kg and Vit. C (L-ascorbyl monophosphate) 550 mg/kg. This should be adequate if given as sole food. However, in the afternoon (adult eventually pregnant) fishes were fed commercially bred crickets. At the breeding facility, crickets had been gut-loaded. Analysis of feeder insects was not performed. It remained uncertain at what moment after delivery to the zoo these crickets were fed to the fishes. A further question is: What happened over weekends? Probably (most) crickets had unloaded their gut contents when fed to *Anableps*.

Wild-caught unrelated fishes were added at irregular intervals.

It would be difficult to confirm presence of a genetic disorder in the four-eyed fingerlings studied.

Histopathological findings are characteristic of deficiencies in vitamin E/Se, and vitamin C known from other vertebrates [33-36].

**We Postulate:** The food offered to the adult fishes and their fingerlings resulted in multiple deficiencies in vitamin E/Se and vitamin C.

#### Acknowledgments

The authors thank J.E. van Dijk, T.S.G.A.M. van den Ingh, and E. Gruys for their help in the interpretation of pathologic changes; Mark de Boer, D. Oonincx, and P. Debatist for their help in clarifying the feeding regimen; Michael Fielding for his help with the English language; G. Grinwis for making the microscopic pictures; M. Bagnat for permitting us to use his drawing and communicating details about the notochord structure.

**Funding:** This project received no external funding.

**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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