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In vitro culture of skin cells from biopsies from the Critically Endangered Chinese giant salamander, *Andrias davidianus* (Blanchard, 1871) (Amphibia, Caudata, Cryptobranchidae)

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***Abstract.*—We established a primary skin cell culture of the Critically Endangered Chinese Giant Salamander, *Andrias davidianus*, from small biopsies using minimal invasive methodologies. Biopsies were taken from three animals simultaneously with assessment of two biopsy sampling techniques using samples from the tail tip. Cell culture was performed in a wet chamber at room temperature. Several culture media and supplementations were tested as well as culture containers and surface coatings. The handling of *A. davidianus* in a landing net, without transfer out of the tank, allowed easier biopsy withdrawal. Best outgrowth of cells from explants was achieved in 60% DMEM/F12 medium with supplementation. Cells started to grow out as monolayer within the first 12 hours, and after three weeks formed pigmented multilayers, then died after 10 weeks. Primary cultures of *Andrias* skin cells, as well as other amphibian primary cell cultures, can be used in future studies to evaluate effects of disease, pollution, regeneration, wound healing, and could provide cells for use in reproduction technologies such as cryopreservation to preserve gene lines in this and other Critically Endangered species with minimal harm to the animals.**

Key words. Caudate cell culture, skin tissue explants, skin biopsy, biopsy withdrawal, amphibian skin cell culture, regeneration, wound healing

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Introduction

The Chinese giant salamander (*Andrias davidianus*) is the largest extant amphibian, with a total length of up to 180 cm. Together with the Japanese giant salamander (*A. japonicus*) from central and southern Japan, and the North American Hellbender (*Cryptobranchus alleganiensis*), these species form the sole members of the giant salamander family Cryptobranchidae, which is thought to be a basal family among caudate amphibians (Gao and Shubin 2003; review Browne et al. 2011). This family might be a survivor of a lineage that was already present in the Jurassic (Gao et al. 2003). The Chinese giant salamander is widespread in central, south-eastern and southern China, although its range is now very fragmented. The species inhabits streams and rivers in mountainous forested areas, at elevations from 100 to 1,500 m above sea level. Once common, the species has declined catastrophically over the last decades in their natural habitats while millions of these animals are bred in farms. Wild harvesting for human consumption is a major threat to *A. davidianus*, along with habitat destruction and degradation (IUCN 2012). Consequently, *A. davidianus* is now very rare in nature. *Andrias davidianus* is listed in Appendix I of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) and is also listed as Critically Endangered on the IUCN Red List of Threatened Species (IUCN 2012).

Research on diseases and other issues in salamanders, including *A. davidianus*, often involves sacrifice of the animals at the end of the experiments (e.g., Geng et al. 2011). An alternative to whole animal experiments that would minimize destruction of the animals is the use of *in vitro* cell cultures. Such assays have already been described for fishes. For example, Estepa et al. (1993) described a cell culture model to study the viral haemorraghic septicaemia virus in fin cells of rainbow trout *Oncorhynchus mykiss* (Estepa 1993). For this assay primary cultures from tissue explants of trout fins were established and infected with the virus *in vitro*.

The purpose of the present study was to determine whether it is possible to establish primary *in vitro* cultures of the skin cells of *A. davidianus* from small biopsies of tail tip tissue. Various cell culture media, surface

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Fig. 1. Biopsy method one performed in the zoo of Berlin. The animal was housed in an exhibition tank. It was captured and transferred in a tub, resulting in aggressive reactions of the animal, which made the biopsy procedure very difficult. A: animal housing. B: animal transferred into tub. C: handling of the animal to perform biopsies.

coatings and types of plastic containers were checked for cell outgrowth and long term survival. We find that this technique could serve as a feasible alternative to studies that require the destruction of individual animals.

Material and Methods

Three adult *A. davidianus* were used for this study. Sexing was done via ultrasound. One male was housed at the Berlin Zoo in an aqua-terrarium (L340 × W160 × H220 cm) with 50 cm water depth with shelter and decorative objects provided (Fig. 1A). Water temperature is 20 °C and water quality maintained by a sand-pressure filter, and partial daily, and complete weekly water changes. The remaining two adult *A. davidianus* were housed at the Cologne Zoo Aquarium. The couple is held in two concrete tanks (each L150 × W190 × H60 cm) with 50 cm water depth. The water (flowing water system) is connected to a cooling system and an external filter (OASE pond filter, Type Biotec Screenmatic) with a capacity of 10,000 L/h. Water parameters are as follows: temperature 20 °C, pH 7.3, conductivity 740 µS, carbonate hardness 7, and total hardness 16. Illumination is provided by T 26 fluorescent tubes (3 × 58 Watt). Tank roofing consists of stainless steel fence (1 cm mesh size), with one half being shaded each by styrofoam mats. Both tanks can be connected through a sliding gate (W60 × H60 cm) consisting of stainless steel wire (1 cm mesh size). The ground substrate consists of gravel and sand mixture with large roots. As hiding possibility, each tank contains a shelter (female tank: L80 × W50 × H50 cm; male tank: L125 × W50 × H50 cm) with entrance in front and exit at the rear side (each opening arched, W36 × H18 cm). Another adult male (not used for this study) is held in a tank in the public area of the Cologne Zoo Aquarium (L350 × W126 × H85 cm; temperature 14 °C, pH value 7.3, conductivity 668 µS, carbonate hardness 7, and total hardness 16; illumination: HQI spotlight, 400 Watt).

Biopsies

In order to keep the biopsy procedure as stress-free and efficient as possible two methods were tested. Method one (conducted at Berlin Zoo): 1) capture of the salamander, and 2) placing it in a tub with water and then taking biopsies from the tail tip (Fig. 1). Method two (conducted at Cologne Zoo): 1) Capture of the salamander in a landing net, and keeping it in its housing tank and taking biopsies (Fig. 2). Minimally-invasive biopsies were performed by using biopsy punches (Stiefel GmbH, Coral Gables, USA), with 4 and 6 mm biopsies taken from the tail tips of two males (Berlin

Fig. 2. Biopsy method two performed in the zoo of Cologne. Animals remained calm during the whole procedure and showed no reactions regarding handling of their tails. A and B: capture of the animal in a landing net. C and D: biopsy procedure at tail tip by use of biopsy punches. E: tissue inside a punch. F: transfer of tissue in tube with amphibian ringer solution for rinsing.

Table 1. Contents of Modified Amphibian Ringer Solution.

NaCl	100 mM
KCl	1.8 mM
$MgCl_2$	1 mM
$CaCl_2$	2 mM
HEPES	5 mM

and Cologne Zoo) and one female (Cologne Zoo). The procedure was performed without anesthesia as pain of biopsy is negligible, and consequently the risks of anesthesia too high. The procedure was classified as minimally invasive and performed in consent with the veterinary commissioner of the Cologne Zoo and the zoo's veterinarians. Giant salamanders are noted for their regenerative capacity, and consequently wound medication was not performed.

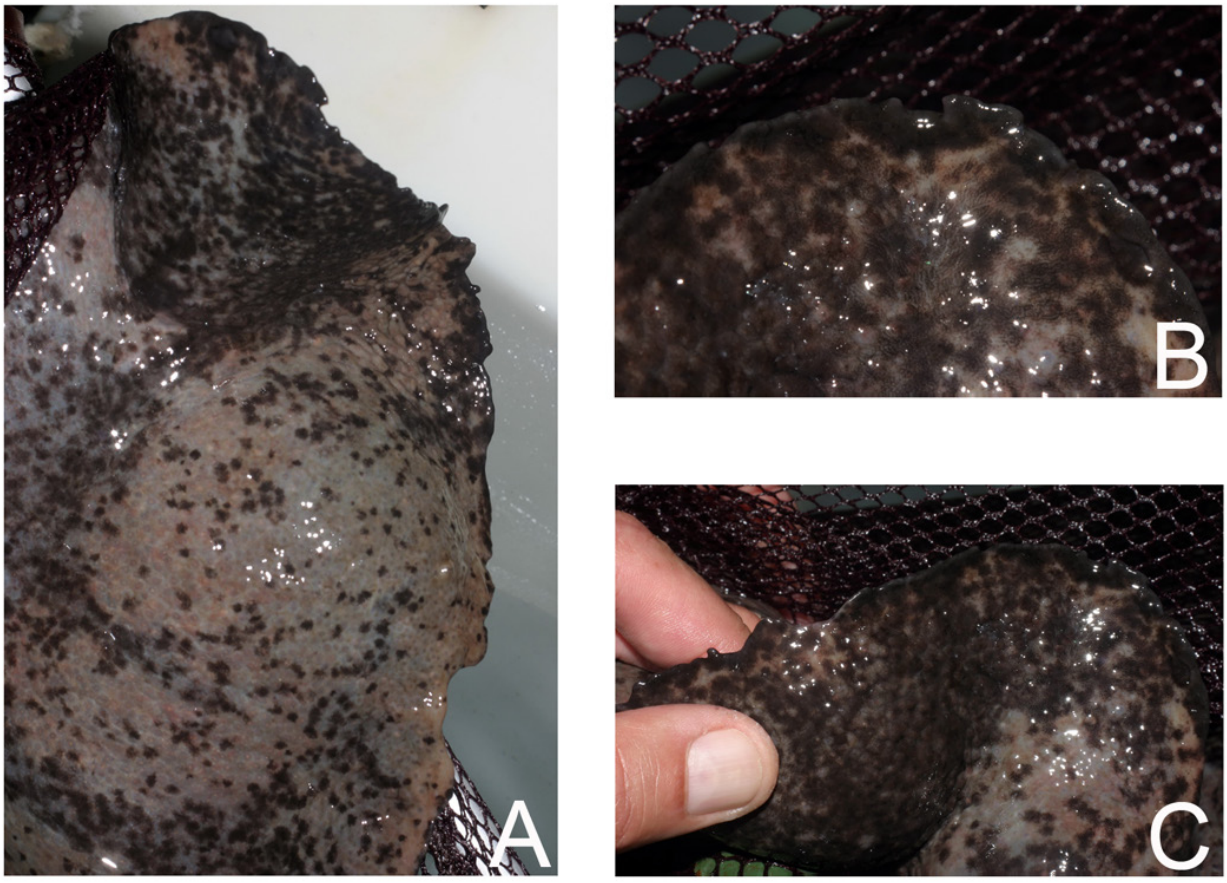

Fig. 3. *Andrias* tails nine months after biopsy procedures. Lost tissue was completely regenerated without scar formation or dyspigmentations. A: overview of tail. B and C: detail of tail tip.

Table 2. Cell culture media, supplements and coatings. Green highlight: optimal conditions for culture of *Andrias* skin cells.

Medium	**Supplements**							**Coating**			
	ITS	Sodium-P	NEA	A2P	P/S	Genta	HEPES	Collagen	PLL	FS	none
Williams Medium E	1%	1 mM	1%		50 U/ml	0.1 mg/ml	+				
	1%	1 mM	1%		50 U/ml	0.1 mg/ml			+		
	1%	1 mM	1%		50 U/ml	0.1 mg/ml				+	
	1%	1 mM	1%		50 U/ml	0.1 mg/ml					+
Leibovitz L-15	1%	1 mM	1%		50 U/ml	0.1 mg/ml	+				
	1%	1 mM	1%		50 U/ml	0.1 mg/ml			+		
	1%	1 mM	1%		50 U/ml	0.1 mg/ml				+	
	1%	1 mM	1%		50 U/ml	0.1 mg/ml					+
DMEM/F12	1%	1 mM	1%		50 U/ml	0.1 mg/ml		+			
	1%	1 mM	1%		50 U/ml	0.1 mg/ml			+		
	1%	1 mM	1%		50 U/ml	0.1 mg/ml				+	
	1%	1 mM	1%		50 U/ml	0.1 mg/ml					+
	1%	1 mM	1%	50 µg/ml	50 U/ml	0.05 mg/ml	5 mM				+

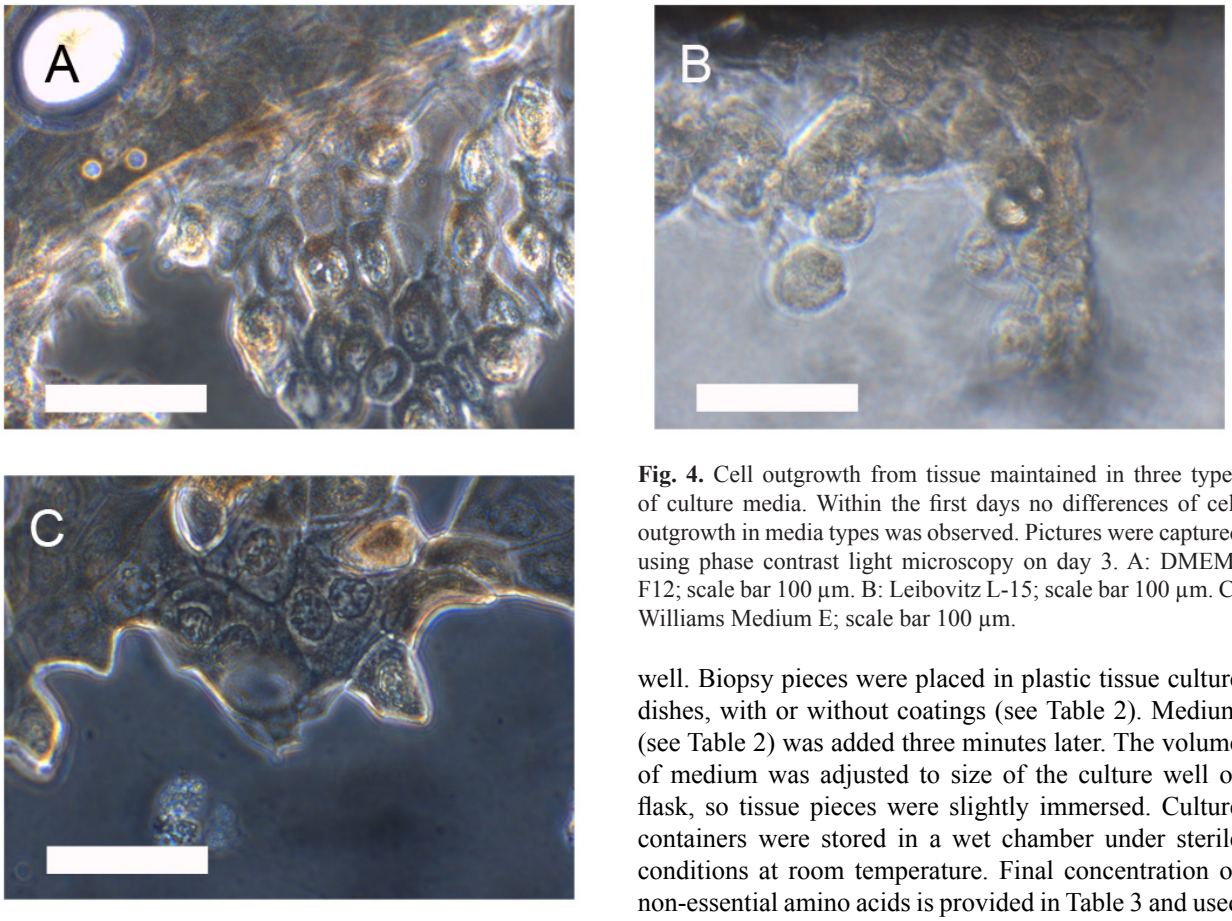

Fig. 4. Cell outgrowth from tissue maintained in three types of culture media. Within the first days no differences of cell outgrowth in media types was observed. Pictures were captured using phase contrast light microscopy on day 3. A: DMEM/F12; scale bar 100 µm. B: Leibovitz L-15; scale bar 100 µm. C: Williams Medium E; scale bar 100 µm.

Tissue Preparation

To reduce microbial contamination of cell cultures, biopsies were rinsed in 60% (v/v) PBS (phosphate buffered saline) or Amphibian Ringer Solution (see Table 1). These were salt solutions adapted to the osmolarity of amphibian cells, at pH 7. The solutions were supplemented with 50 U/ml penicillin/streptomycin (Biochrom) and 0.05 mg/ml gentamicin (Biochrom) to further support reduction of microbial contamination. Samples of one male *A. davidianus* (Berlin) were transported in cell culture media without supplementation for four hours. Samples of one male and one female animal (Cologne) were rinsed carefully, directly processed for cell culture without use of a cell culture workbench and after adherence transported to their storage place within three hours.

Biopsies were processed by cutting them into small (1-2 mm) pieces. As only small tissue samples were available, we decided to perform cell culture in small containers. The choice was between flasks that could be sealed thus making them suitable for transport of the culture from the zoo to the lab and multi well plates which are commonly used for cell culture assays. So 25 cm^2 tissue culture flasks were used especially for the starting cultures and 24 and 12 well plates were tested as well. Biopsy pieces were placed in plastic tissue culture dishes, with or without coatings (see Table 2). Medium (see Table 2) was added three minutes later. The volume of medium was adjusted to size of the culture well or flask, so tissue pieces were slightly immersed. Culture containers were stored in a wet chamber under sterile conditions at room temperature. Final concentration of non-essential amino acids is provided in Table 3 and used abbreviations and suppliers in Table 4.

Culture Containers

Following containers were examined for cell culture:

- 12 well plates, attachment surface of 3.6 cm^2/well (#92012, TPP, Trasadingen, Switzerland).
- 24 well plates, attachment surface 1.9 cm^2/well (# 92024, TPP, Trasadingen, Switzerland).
- Microflask, attachment surface 10 cm^2 (#91234, TPP, Trasadingen, Switzerland).
- Miniflask, attachment surface 25 cm^2 (# 90025 and 90026, TPP, Trasadingen, Switzerland).
- 24 well plates, attachment surface 1.9 cm^2/well (#CC7682, Cyto One, USA).
- Miniflask, attachment surface 25 cm^2 (#7.690, Greiner Bio One, Frickenhausen, Germany).

Depending on manufacturer´s production processes adhesion surfaces of the containers might be treated differently (e.g., plasma treatment of surfaces with varying protocols), resulting in varying adhesion conditions. As from mammalian primary cell culture is known that not every cell type adheres on every type of culture plastic, containers of various manufacturers were examined for cell culture of *Andrias* skin tissue explants.

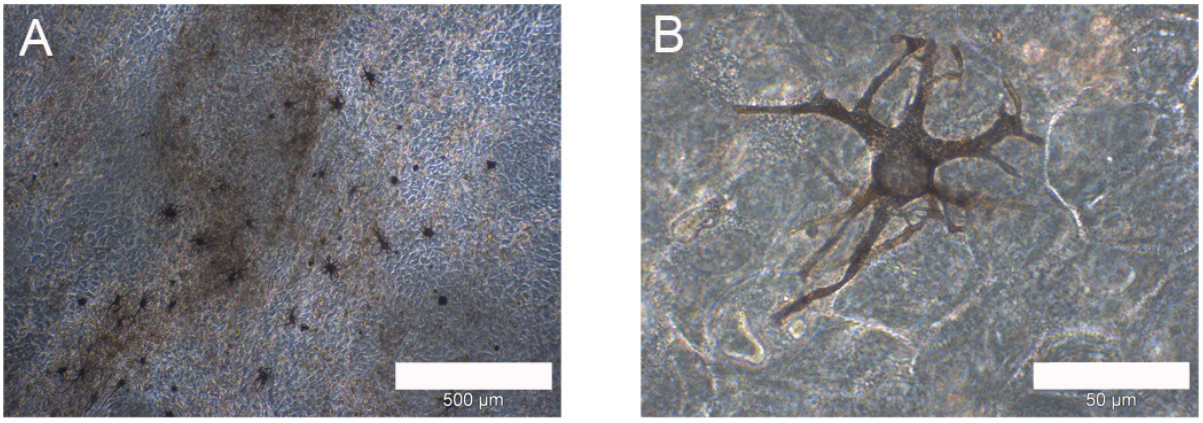

Fig. 5. Melanophores migrating from tissue. Skin epithelial cells and melanophores after two weeks of cultivation. The morphology of resident *Andrias melanophores* (A and B) appeared similar to observations of Billingham et al. in cytology of pigmented guinea pig skin. (C). Pictures were captured using phase contrast light microscopy. A: overview, migrating melanophores appeared rounded, whereas resident appear in the typical dendritic form; scale bar 500 µm. B: resident melanophore; scale bar 50 µm. C: resident melanophore (Billingham 1948).

Table 3. The final concentrations of non-essential amino acids and ITS in cell culture µg/ml.

L-alanine	8.9
L-asparagine*H_2O	15
L-aspartic acid	13.3
L-glutamic acid	14.7
Glycine	7.5
L-proline	11.5
L-serine	10.5
Insulin	10
Transferrin	5.5
Selenium A	0.0067

Media, Supplements and Coatings

All cell culture media were diluted to 60% (v/v) with sterile distilled water to achieve appropriate osmolarity. Media, supplementations and plastic coatings are listed in Table 1.

Cell culture material was coated by dropping solutions on the surfaces and drying under sterile conditions under a workbench, followed by three rinsing steps with sterile distilled water. Afterwards surfaces were dried again under sterile conditions. Coated surfaces were stored under sterile conditions at 4 °C for a maximum of one week. Media were changed twice a week. Cell outgrowth was digitally photographed with an inverse microscope and Cell D software (Olympus).

Results

Biopsy in A. davidianus

Method one resulted in aggressive reactions of the male that made taking of the biopsy difficult (Fig. 1). With method two both salamanders remained calm and did not react to the biopsy taking, which took less than five minutes (Fig. 2). Directly after biopsy the wounds bled sparsely or not at all, and inflammation and/or infection of the wounds did not occur. Healing took about two months; the lost tissue was completely regenerated without scar formation (Fig. 3).

Cell Culture

Cell culture was performed in a wet chamber at room temperature. Initially, technical difficulties had to be overcome resulting from low rates of adherence of the tissue fragments. In 12 and 24 well plates and microflasks, the tissue fragments adhered only in small proportions (5%), whereas more than 80% of the fragments adhered on the plastics of both types of miniflasks (Greiner and TPP). Cells started to grow out from adhered tissue under all culture conditions within 12 hours (Fig. 4). Beside skin epithelial cells also melanophores grew out. The melanophores appeared rounded during migration processes whereas resident cells showed typical dendritic morphology (Fig. 5).

Surface coatings did not result in better adherence or enhanced outgrowth. Interestingly, outgrowth from the female tissue appeared to be faster and spatially extended more than those from the males. Whether this observation is a general phenomenon or just occasional should be

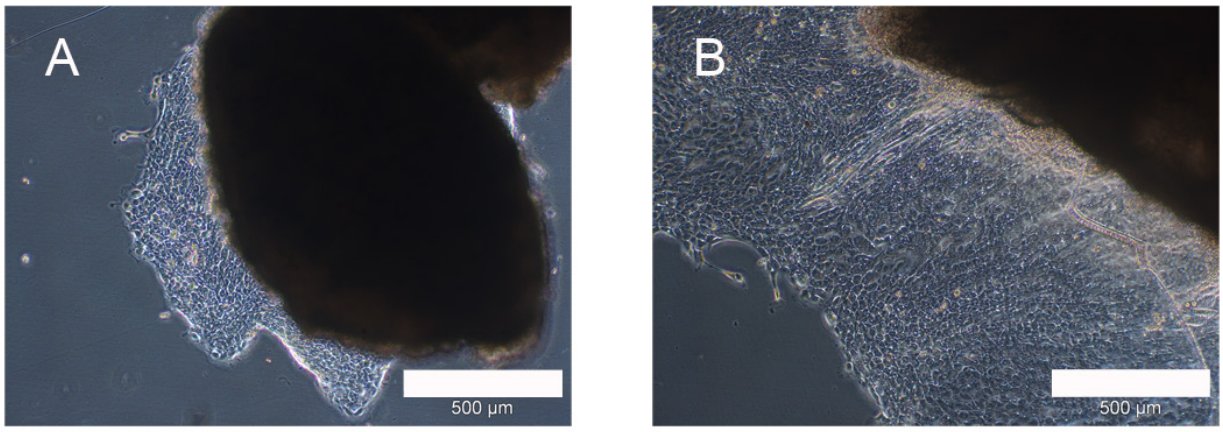

Fig. 6. Comparison of male (A) and female (B) tissue after three days of cell culture. Note that more outgrowing cells were observed in the female samples. A: male tissue at day three; scale bar 500 µm. B: female tissue at day three; scale bar 500 µm.

examined in further studies with higher numbers of tested individuals. In our study female cells grew out earlier and covered greater areas indicating faster rates of migration (Fig 6). Additionally, male cells became senescent earlier.

Influence of media conditions was tested in long-term culture. Cells in Leibovitz or WilliamsE cell culture media survived only for two weeks whereas cells with DMEM/F12 survived for 10 weeks. Cells grown in DMEM/F12 with full supplementation (see Table 2, green highlight) generally showed best results (Fig. 7). Cells grew out, formed complete monolayers and started to form tissue-like structures with pigmentation (Fig. 7 and 8). After six weeks multi nucleic cells occurred more frequently (Fig. 9), these cells stopped growing and finally died after 10 weeks. Dead cells broke away from the adhesion surface and floated in big sheets in the containers. Medium supplementation with HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) resulted in pH stabilization (visualized by phenol red indicator in cell culture media). Without this buffer medium's pH changed after less than one hour in the wet chamber as CO_2 fumigation was not available. With HEPES pH remained stable for up to two days. This short time of stability was caused by the low concentration of HEPES (5 mM) and small volumes of medium applied to the cells. Usually a concentration of 10 mM is used to stabilize media, but this concentration was found to be harmful to the cells of the giant salamander.

Problems with contamination by a fungus (white appearance, no determination of species performed) occurred in cell culture from one male animal (Cologne) and were treated with amphotericine B (Biochrome). This treatment stopped fungus growth, but cells started to age after two days of antifungal treatment. The cultures of the female (Cologne) and the other male (Berlin) tissues remained uncontaminated during the culture process. Repeated preparations from further biopsies of Cologne animals at later time points resulted again in fungal contaminations of male cultures.

Discussion

The large size and weight of adult *Andrias davidianus* make handling of the animals difficult and cause stress and possibly injury for both animals and researchers (e.g., bites, Beckstein 2009). To minimize such risks, we recommend using a landing net to restrain the animals in the housing tank for biopsy procedures as the animals stayed absolutely calm and apparently oblivious to the procedure (cf. Nickerson 2003; Mutschmann 2009).

We could find no literature concerning the cell culture of *A. davidianus* or any other cryptobranchid species in Western literature, or from correspondence through Chinese literature. Based on the cold freshwater physiological conditions experienced by *A. davidianus*, cell culture could be expected to be most successful with lower temperatures than with mammalian cells. Other conditions to consider with the establishment of *A. davidianus* cell cultures, in respect to those of mammals, are a lower osmolarity of body fluids in *A. davidianus* (Albert et al. 1987; Chernoff et al.1990), and particular cell culture coatings for optimal cell adherence and proliferation, as shown with *Xenopus laevis* and *Ambystoma mexicanum* primary cells (Nishikawa et al. 1990; Chernoff et al.1990). We assessed the use of different cell culture containers and various treated plastics (according to manufacturer's datasheets) combined with various media conditions and surface coatings.

We found that the size of cell culture containers was important for the successful outgrowth of cells, and tissue pieces were more likely to stick when small flasks were used instead of multiwell plates. This might be explained by the tendency of small pieces of tissue to float on the surface of solutions toward the containers wall thus preventing adhesion to the bottom of the container.

Cells from multicellular organisms communicate with each other by release of messenger substances into the extracellular fluids, e.g., the culture medium, or by direct cell-cell contacts. To accomplish sufficient concentrations

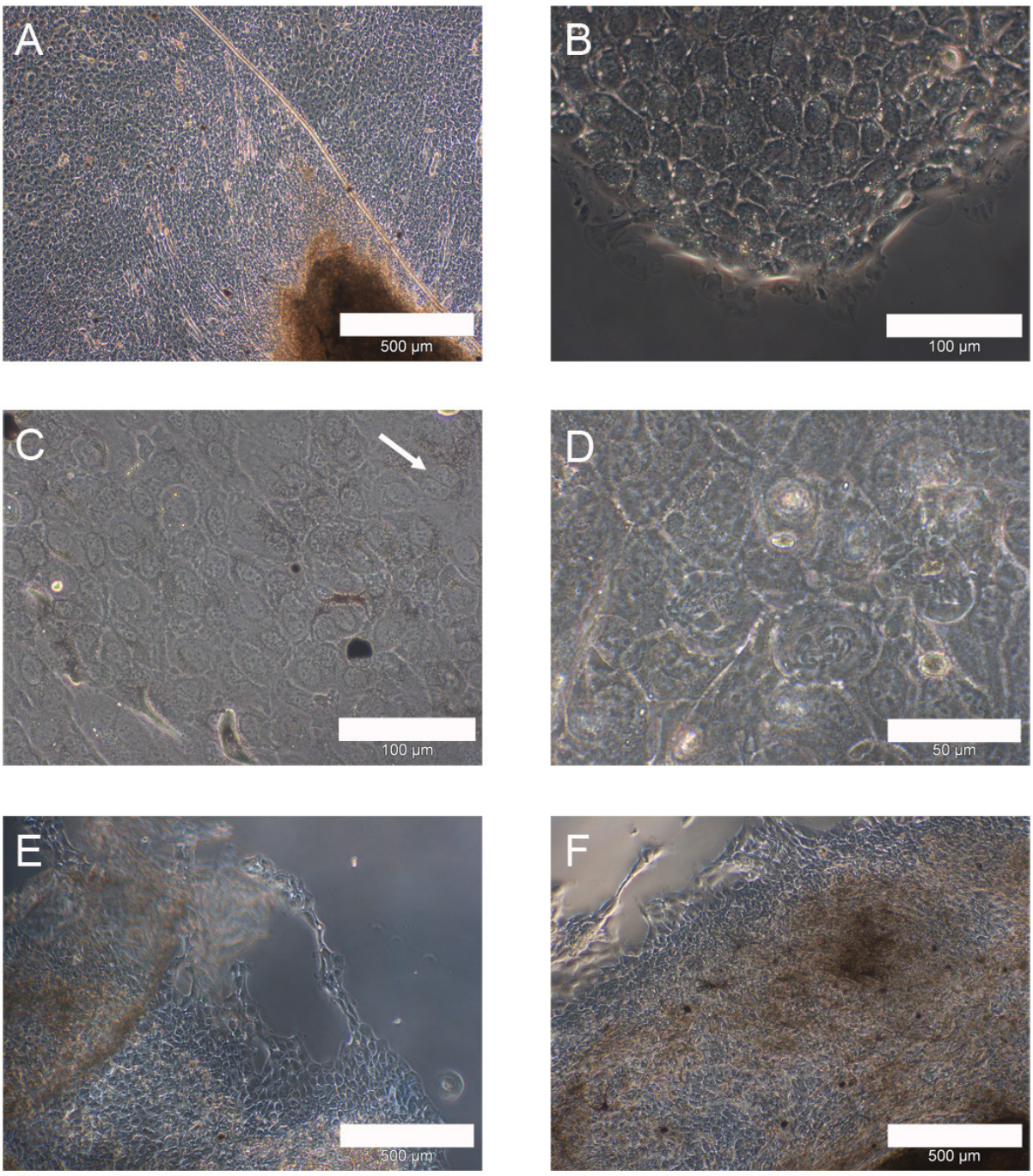

Fig. 7. Picture time line of cell outgrowth in DMEM/F12 with full supplementation. Images in overview and detail show representative examples of long term outgrowth of cells under full supplementation. Cells grew out in dense layers (A). At the migration front cells filopodia formation was observed (B). Outgrowing cells proliferated (C, indicated by arrow). No visual evidence for senescence was observed at day 18 to 25 (D, E, and F). After three weeks cells started to form pigmented tissue-like structures (E and F). Pictures were captured using phasecontrast lightmicroscopy. A: cells at day three; scale bar 500 µm. B: cells at day three; scale bar 100 µm. C: cells at day seven; scale bar 100 µm. D: cells at day 18; scale bar 50 µm. E: cells at day 21; scale bar 500 µm. F: cells at day 25; scale bar 500 µm.

of bioactive molecules by cellular release of substances like growth factors (e.g., vascular endothelial growth factor, keratinocyte growth factor, fibroblast growth factor), enzymes (e.g., lipoxygenases) and cytokines (e.g., interleukines) to their culture medium, low volume for small cell numbers is recommended. Too low concentrations of these substances lead to cell death *in vitro* as cells are missing paracrine stimulation. So the choice of cell culture container size means a balancing act between low surface curvature (implying use of greater culture containers) and low medium volume (implying use of smaller culture containers).

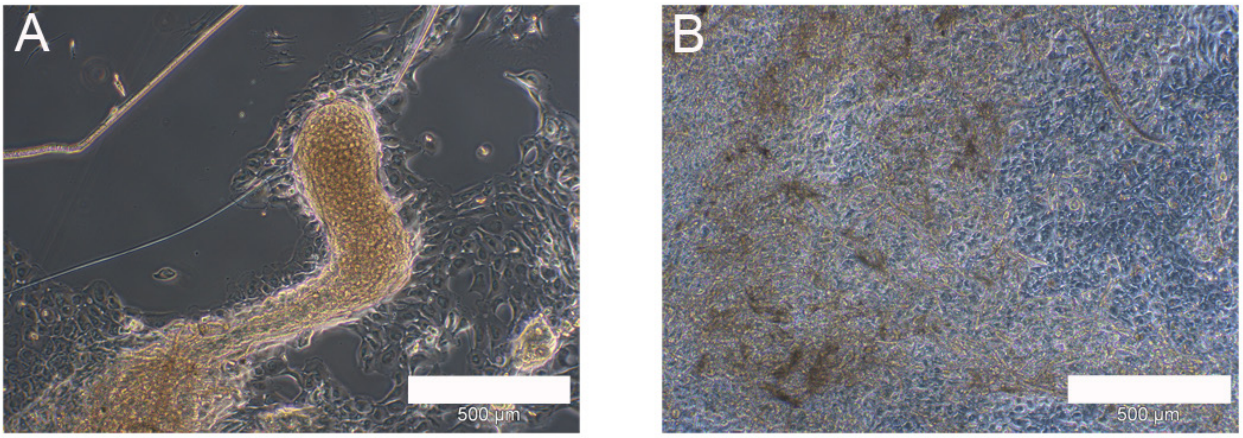

Fig. 8. Multilayer formations after six weeks of cultivation. Outgrowing cells tended to form pigmented multilayers with tissue-like appearance which became thicker with prolonged cultivation time. A: tissue-like structure after six weeks; scale bar 500 µm. B: tissue-like structure with pigmentation after six weeks of cultivation; scale bar 500 µm.

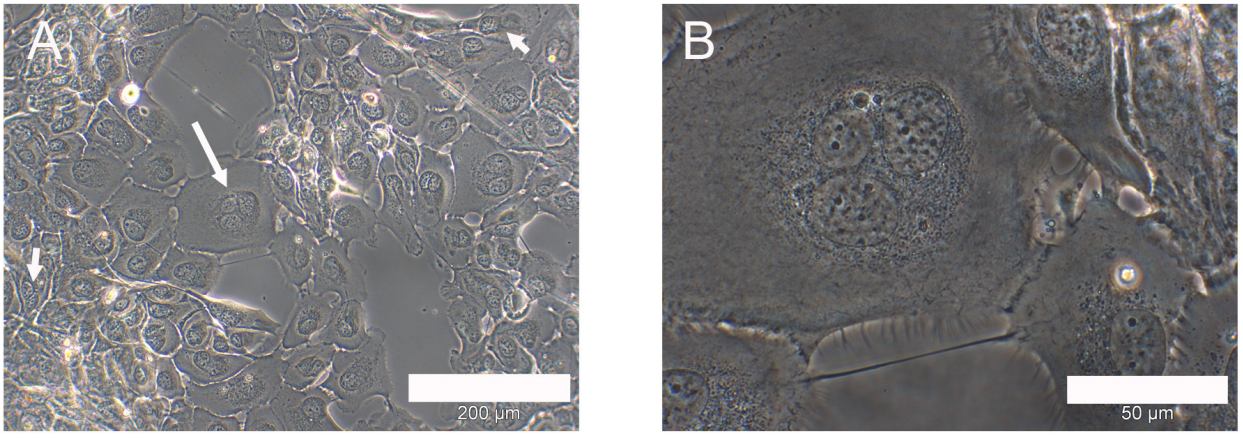

Fig. 9. Cell aging. After six weeks in DMEM/F12 multinuclear cells were observed more frequently. Pictures show representative examples and were captured using phasecontrast lightmicroscopy. A: overview (multinuclear cell indicated by arrows); scale bar 200 µm. B: detail of A; scale bar 50 µm.

Table 4. List of abbreviations and suppliers.

Williams Medium E		PAA, Cölbe, Germany
Leibovitz L-15		PAA, Cölbe, Germany
DMEM/F12		PAA, Cölbe, Germany
Ascorbate-2-phosphate	A2P	Sigma Aldrich, Taufkirchen, Germany
Insuline-Transferrine-Selenium A	ITS	Gibco
Non-essential aminoacids	NEA	Biochrom, Berlin, Germany
Sodium-Pyruvate	Sodium-P	Biochrom, Berlin, Germany
Penicilline/Streptomycine	P/S	PAA, Cölbe, Germany
Gentamicine	Genta	Biochrom, Berlin, Germany
Amphotericine B	Ampho	Biochrom, Berlin, Germany
(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid	HEPES	PAA, Cölbe, Germany
Collagen		Biochrom, Berlin, Germany
Poly-L-Lysine	PLL	Biochrom, Berlin, Germany
Fish Serum	FS	own production from trout blood

The influence of the adhesion surface on adhesion rates, cell migration, cell growth or the culture survival time seems to be negligible as no correlation to the cell culture material or surface coatings was observed. This is contrary to data from the literature describing culture of various amphibian cell types from *X. laevis* and *A. mexicanum* on developmental or regenerative aspects as well as toxicological studies (Albert at al. 1987; Nishikawa et al. 1990; Chernoff et al.1990; Goulet et al. 2003 et al.; Ferris 2010). In those studies cell culture vessel plastics were coated with fibronectin, collagen, matrigel and other matrices to encourage cell adhesion.

As nutrition media MEM, F12, MCDB151 or combination of these diluted to 70% with sterile distilled water were used (Nishikawa 1990). Culture media were supplemented with insulin, transferrin and EGF. Skin explant cultures obtained from *Ambystoma mexicanum* can be grown in 60% DMEM under supplementation with 10% fetal bovine serum and ITS (insulin transferrin, selenium A) (Ferris et al. 2010).

Culture survival appeared to be more dependent on the stabilization of culture medium pH than on surface coatings; mammalian primary cells usually need a stable pH around 7 to remain vital *in vitro*. Cells of *A. davidianus* were very sensitive to the supplementation with HEPES while the commonly used concentration of HEPES of 10 mM was toxic to the cells and led to cell death. A concentration of 5 mM resulted in stabilization of the pH as well as no detectable toxic influence on *A. davidianus* cells. High sensitivity to HEPES was also shown with a blastema model of A. mexicanum (Guelke et al. submitted). Previous publications on amphibian cells did not mention the use of HEPES in the culture media. Alternatively to HEPES, an incubator with CO_2 fumigation can be used to stabilize the pH (Chernoff et al.1990; Nishikawa et al. 1990; Ferris et al. 2010). Without pH stabilization cell outgrowth and survival was greatly reduced in our study as well as in other studies using CO_2 fumigation.

The benefit of the use of antibiotic supplements in amphibian cell culture may be negated by decreased survival. As caudates do not live in a sterile environment and need a certain skin flora, thus a problem rises with the transfer of tissue to cell culture; the culture medium offers good growth conditions for the target cells and simultaneously for microorganisms. Bacteria and fungi accrete faster than the cells and cause cell death by release of toxic substances. In our study, cells tolerated 50 U/ml of penicillin/streptomycin mix (p/s) which is sufficient to avoid infections of already established cultures. Therefore 0.05 mg/ml gentamicin was thus added. The common antibiotic supplementation of cell culture media contains 0.1 mg/ml of gentamicin, but this concentration resulted in early senescence and cell death of *A. davidianus* cells. There is no comparative research in Western scientific publications on the use of antibiotics in amphibian cell culture media.

The fungal contamination of the Cologne Zoos male's cell culture appeared to be from the skin microflora. Contaminations during tissue processing seem an unlikely cause as culture contaminations occurred under a wide range of preparation conditions including sanitized conditions. Further research is planned to identify the type of fungus and to assess its possible influence on outgrowth of cells from the tissue explants. *In vitro* treatments with amphotericin B for this fungus resulted in early senescence and cell death. Causes for this toxic effect remain unclear as amphotericin B (Fungizone) is commonly used in fish and amphibian cell cultures and known to be not toxic to cells so far. There is only one publication mentioning possible toxic effects of amphotericin B (Fungizone) on tadpoles of *Alytes cisternasii* (Martel et al. 2011).

Based on cell morphology we consider that outgrowing cells were skin epithelial cells and melanophores. Migrating melanophores appeared rounded while resident cells showed typical dendritic forms as these cells are from dendritic origin (Rawles 1948; Billingham 1948). In light microscopic imaging melanophores of *A. davidianus* appeared equal to those of guinea pigs shown in the study of Billingham (1948) which are compared in Fig. 5.

Interestingly, cell outgrowth from female tissue appeared to be faster than from male (Fig. 6). As we tested only samples of three animals so far, these observations need to be confirmed by repeating trials with other giant salamanders. From MRL mice it is known that females heal wounds better and faster than male animals due to sexually dimorphic genes (Blankenhorn et al 2003) and also with human cutaneous tissue (Gilliver et al. 2007), however we could find no published information on this phenomenon in fish or amphibians.

Cells did not only form a monolayer as known from primary cells in general, but tended to form pigmented multilayers in long term cultivation (Fig. 7F and 8) after three weeks. Usually mammalian primary cells stop proliferation when reaching confluence *in vitro* due to contact inhibition by cell-cell and cell-substrata interactions (Qi et al. 2008). In contrast most cancer cells or immortalized cell lines are refractory to contact inhibition and can continue to proliferate (Hanahan et al. 2000). Cell cultures from *Xenopus* skin explants only grew out as monolayer stopped expanding after six to eight days (Reeves et al. 1975). This raises the question whether the observed multilayer formation of *Andrias* skin explants could be related to the regenerative capacity of caudate amphibians.

Senescence is a well-known process in mammalian primary cells. Due to their limited proliferation capacity (Hayflick index) mammalian cells become senescent after certain time of *in vitro* cultivation in contrast to immortalized cell lines. Literature regarding life span of amphibian primary cells is limited and described results are ambiguous. While Nishikawa reports ageing

of *Xenopus* skin cells *in vitro* (Nishikawa 1990), Kondo et al. (1983) describes a growth crisis (senescence) in melanophores followed by a spontaneous transformation to an immortalized cell line derived from Rana catesbeiana (Kondo et al. 1983). In our study skin cells became senescent and did not undergo a spontaneous transformation and eventually died.

The creation of an immortal *Andrias* skin cell line could possibly be achieved by: 1) spontaneous transformation of cells as a small number of them undergoes a set of genetic alterations which lead to unlimited life span. This means, however, that very high numbers of primary cells may have to be cultivated over a long period of time until some of them start unlimited proliferation. 2) expression of telomerase reverse transcriptase (TERT) which e.g., is available as eukaryotic expression plasmid from ATCC (MBA-141). The use of method one is well documented in anurans (Kondo et al. 1983) as well as in fishes (review Lakra et al. 2011) while for method two only literature regarding fish cell lines is available (review Lakra et al. 2011).

Conclusion

This study examined the basic needs of primary cultures for *A. davidianus* skin cells raised from small skin biopsies. These cells seem to have no exceptional culture needs when cell culture is performed in a wet chamber except for specific medium osmolarity and pH stabilization with HEPES buffer.

Primary cultures of *Andrias* skin cells, as well as other amphibian primary cell cultures can be used in future studies to evaluate effects of; 1) diseases and effects of medication, 2) toxicity tests of pollutants and other substances as already described for fishes (Dayeh 2005) and anurans (Goulet 2003), 3) for the study of regeneration, and 4) the role of gender specific hormones on wound healing. The use of active or cryopreserved cell cultures, in conservation programs for threatened amphibians is being increasingly recognized. These cells can provide for the banking of cells and organelles, and their genetic material for use in reproduction technologies (Browne et al.). The next steps in the establishment of an *in vitro* cell culture model will be on the one side the development for cryopreservation cells do not have to be immortalized; they can be stored and cultivated we predict as mammalian primary cells.

A further contribution to cryptobranchid conservation of cell lines is their use for establishing of a karyogram based sex determination. Because of the large size of cryptobranchids sexing is often performed by ultrasonic examination, and due to the size of adult *Andrias* is an elaborate procedure. During ultrasonic examination which is usually done without anesthesia, also injury risks, both for animals and human beings, must be considered. Sexing with ultrasound is also most effective during the breeding period, when gonads are distinct and may effect reproduction. Based on the study of Zhu et al. (2002) *A. davidianus* may be distinguished by their sex chromosomes and this technique would enable a new less stressful sexing of these salamanders. Karyotyping also offers the opportunity to screen the animals for chromosomal aberrations to distinguish salamanders that may be unsuitable for use in conservation breeding programs. However, skin cell karyograms can only provide insights into chromosomal aberrations of somatic cells and not those induced by failures in the germ line. Examination of wound closure processes resulting from biopsy withdrawal *in vivo* and cell outgrowth *in vitro* could give information about the regenerative capacities of *A. davidianus*. Using cell culture models for *A. davidianus* research would reduce the number of experimental animals and provide new research horizons and benefit conservation breeding programs.

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