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Amphibian cell lines: Usable tissue types and differences between individuals within a species

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***Abstract.*—Amphibian conservation efforts have never been more imperative than they are now, such as by preserving genetic material through establishing cell lines. This study describes a successful protocol for culture of cells from three tissues (whole limb, tongue, toe clip) taken from five individuals of the Asian Common Toad (*Duttaphrynus melanostictus*) and using 100% Cellgro Minimum Essential Medium (MEM) Alpha 1 X (Fisher Scientific) as basic medium alone and in combination with three different supplements (ITS, FGF, and 2-mercaptoethanol). Real-time cell analysis was used to test for differences in cellular growth patterns among individuals and media. Cell lines were established from all three tissue types, with the tongue tissue giving the highest success rate and a more stable growth pattern. Real-time cell analysis displayed no significant differences between the tested media; although toe clip tissue tended to function better with one of the media including 2-mercaptoethanol. Growth patterns were consistent within each individual but varied among individuals. The knowledge provided by this study can be used to further improve the protocols, storage, and safeguarding of viable genetic material from amphibians.**

Keywords. Cell culture, growth conditions, xCELLigence, real time analysis, amphibian, biobanking

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Introduction

Cell culture is a routine and widespread procedure today (Davis 2011; Freshney 2016), but mammals have received far more attention, whereas research on lower vertebrates and invertebrates is very limited (Masters 2003; Freshney 2016). There is a global amphibian crisis triggered by various anthropogenic factors such as disease, climate change, pollution, and various other factors, so amphibians are now considered as the most threatened group among vertebrates (Ficetola et al. 2015; Ceballos et al. 2015; Zimkus et al. 2018; Strand et al. 2020). Therefore, conservation efforts that involve preserving genetic material such as through cryopreservation and biobanking have never been more imperative. Biobanking genetic material, such as cell lines, provides an expandable resource of high-quality biological material with various applications. These range from tissue engineering and the replacement of live animals in toxicology and virology studies to the broad application within conservation as genetic rescue and population enhancement through captive breeding or reintroduction programs (Yuan et al. 2015; Freshney 2016; Zimkus et al. 2018). Successful work on cell lines from amphibians has been reported using different tissues such as skin, foot, tongue, and eye (Zimkus et al. 2018), but no direct comparisons have been made between tissue types. Furthermore, the current research on amphibian cell lines is limited to a few families and species, and most of these studies are more than 20 years old, indicating the severe lack of research on amphibian cell lines (Clothier et al. 1982; Slack et al. 1990; Tata et al. 1991; Okumoto 2001; Groot et al. 2012; Zimkus et al. 2018). The aim of this study was therefore to evaluate and validate the practicality and optimal growth conditions using different tissue types, as well as detecting differences among cell lines from individuals, within one amphibian species. We succeeded in establishing cell lines from three different tissue types using real-time cell analysis to test growth patterns and preferences according to media type. We also demonstrated variation in growth patterns among individuals within a single amphibian species.

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Table 1. Detailed description of biopsy medium composition including basic medium and supplements for different tissue types.

Basic medium	Supplements	Type of tissue
Complete Alpha MEM medium supplemented with 1% antibiotic-antimycotic 100X (P/S/F) (10,000 units/mL of Penicillin, 10,000 ug/mL of Streptomycin, and 25 ug/mL of Fungizone (Amphotericin B)) (Gibco®)(Houck et al. 2017)	Additional 15 ug/mL Fungizone (Amphotericin B) (Gibco®) + 0.01% Normacin (InvivoGen 500 mg)	Limb
	No supplements	Tongue
	Additional 15 ug/mL Fungizone (Amphotericin B) (Gibco®) + 0.01% Normacin (InvivoGen 500 mg)	Toe clip

Materials and Methods

Animals

Experimental tissues were recovered from five individuals of the Asian Common Toad (*Duttaphrynus melanostictus*) that underwent euthanasia following serious injury. Dead animals were stored at 4 °C until sampling within 24 h. All work was carried out in accordance with The Code of Ethics of the World Medical Association of *The Declaration of Helsinki*, and it complied with the *EU Directive 2010/63/EU for Animal Experiments*.

Biopsy and culture media

Biopsy medium was prepared according to (Houck et al. 2017), see Table 1, consisting of a basic medium with or without supplements.

Culture media (see Table 2) was prepared in four versions (Control, ITS, FGF, and 2-mercaptoethanol). Whole limb and tongue tissues were tested with Control, ITS, and FGF media, whereas toe clip tissue was tested with Control, ITS, and 2-mercaptoethanol media. Media including 2-mercaptoethanol was tested due to successful parallel studies in other species, testing the effect of the reducing agent 2-mercaptoethanol.

Pre-treatment and primary cell culture

The three different tissue types were collected immediately after euthanasia, after the relevant areas were first washed manually with 70% v/v ethanol (20 s) and sterile phosphate-buffered saline (+ $CaCl_2$ and $MgCl_2$) (PBS) (20 s) (Gibco®). Whole limb tissue was obtained from the hind legs, while toe clips were from all four limbs, using both scalpel and scissors, and tongue tissue was obtained with forceps and scissors. All tissue pieces (5 mm were stored in biopsy media [Table 1]) at 4 °C before further processing. Tissues were prepared using the explant method where minced tissue pieces are attached in a culture flask (Houck et al. 2017). First, the tissue pieces were cleansed by immersion: in 70% v/v ethanol for 30 s, in PBS three times [fresh batch each time (20 s)], in antibiotic-antimycotic (Gibco®, Life Technologies, Rockville, Maryland, USA) for 20 s, in PBS once, in Gentamicin (Sigma-Aldrich, Inc, St. Louis, Missouri, USA; 50 mg/mL) for 20 s and then washed in PBS. Tissue pieces were then placed in PBS and minced (3 mm in size) with forceps, thereafter each tissue piece was placed in a 12.5 cm^2 culture flask for 5 min (attachment) before adding 2 mL of media A–D. The cell culture flasks were then placed at 28 °C with 5% CO_2 with

Table 2. Detailed description of culture media composition including basic medium and supplements for different tissue types.

Culture media	Basic medium	Supplements	Type of tissue
Control medium	100% Cellgro Minimum Essential Medium (MEM) Alpha 1 X (Fisher Scientific) supplemented with 10% foetal bovine serum (Gibco) and 1% Penicillin-Streptomycin-Glutamine (29.2 mg/mL L-glutamine, 10,000 units/mL Penicillin and 10,000 µg/mL Streptomycin sulfate (Gibco®) (Houck et al. 2017). Plus 0.01% Normacin (InvivoGen 500 mg)	No supplement	Limb Tongue Toe clip
ITS medium		20 µL/mL ITS (100 µL Insulin 10 mg/mL + 100 µL Transferrin 5.5 mg/mL + 10 µL selenite 20 µg/mL) (Sigma)	Limb Tongue Toe clip
FGF medium		20 ng/mL FGF	Limb Tongue
2-mercaptoethanol medium		0.1 mM 2-mercaptoethanol (Pharmacia Biotec)	Toe clip

the media being changed every four days (in the first two weeks only half the volume was changed, thereafter the entire volume). At 90% confluency (assessed in a Leica DMC 4500 microscope) cells were passaged, i.e., harvested by adding 0.5 mL of TrypLE (Gibco®), before being split into new cell culture flasks at a ratio of 1:2 to 1:3 and incubated under the conditions described above.

Cryopreservation and recovery

Cells were passaged five times and then harvested by adding 0.5 mL of TrypLE (Gibco®). After cell detachment, 8 mL of Hanks Balanced Salt Solution (1X) (HBSS) (Gibco®) was used to rinse out the cells. After centrifugation, the supernatant was removed and the cells were resuspended in freezing medium (Alpha MEM media supplemented with 10% DMSO) (Houck et al. 2017). Cell suspensions (0.5 mL) were kept in 1 mL cryo vials (Nunc®, Cat: 375353 Thermo Scientific, Roskilde, Denmark) and placed in a CoolCell® (Sigma-Aldrich, Inc., St. Louis, Missouri, USA) at -80 °C for 24 h before being transferred to liquid nitrogen (LN2) for long-term storage. Thawing of cell lines was performed in a water bath at 30 °C for 1–2 min, and cells were thereafter transferred to 25 cm^2 cell culture flasks containing 4 mL of one of the four media (Table 2) before being cultured at 28 °C. Cell proliferation post-thaw was observed and followed in all cell lines used. Recovery of cell lines was followed daily but no negative impact of cryopreservation in terms of growth pattern was observed in any of the cell lines.

Evaluation of growth conditions by real-time cell analysis

The xCELLigence® Real-time Cell Analysis System (RTCA SP Bundle, ACEA Biosciences) was used for real-time measurements of cell proliferation and adhesion, following the instruction manual. The xCELLigence® System measures cell index (CI), which represents the change in impedance divided by the background value. The CI provides an indication of both adhesion and proliferation rates. Cells that are strongly adherent will reach a maximum CI of 10–15, whereas a CI of 1–4 is defined as weak and a CI of 5–10 is considered moderate to strong (Raker et al. 2011; Kho et al. 2015). All thawed cell lines were seeded in 25 cm^2 cell culture flasks (Nunc® Easy flask) containing 4 mL of media and incubated at 28 °C. Cell numbers were counted with the CountessTM II FL (Applied Biosystems) to ensure a seeding density of 10,000 cells/well, which was chosen based on parallel experiments on amphibian cells (Strand et al. 2021). After seeding, the plate was incubated at 28 °C with 5% CO_2 for CI readings every 30 min; the medium was changed after 72 h. As controls, wells with DMEM culture media and no cells were included and displayed CI values of zero throughout the experiment. Data were collected and analyzed during the experiments by the RTCA software (Agilent, Santa Clara, California, USA) (Raker et al. 2011; Kho et al. 2015). Throughout the text, (CI) will be described as CI/h to specify the CI at a specific time.

Statistical Analysis

Differences in Mean Cell Index values among individuals were assessed with one-way ANOVA, then Tukey's test was used to test pairwise difference between individuals. The PAST software program (https://www.nhm.uio.no/english/research/infrastructure/past/) was used for all statistical analyses.

Results

Growth patterns of cells from different tissue types

1) Growth of cells in different culture media (Table 3)

Growth patterns were tested among the three different tissue types using four different media. The following growth pattern categories were chosen: (i) No cell growth observed within 12 weeks; (ii) Fungi and/or bacteria were detected at some point during culture, so the culture could not be salvaged and was lost; (iii) Cell growth was observed, but the culture stopped at 10–100 cells; (iv) Cell growth continued throughout the planned culture period, so the cell line could be cryopreserved.

Whole limb tissue: Cell growth was observed in 51% of the replicates, representing four individuals. Of the replicates, 29% and 2% were lost due to fungal and bacterial contamination, respectively. From four different individuals, 38% of the replicates reached cell line stage (a minimum of four times) and were cryopreserved. When using Control, ITS, and FGF media, 40%, 40%, and 33% of the replicates resulted in cell lines, respectively.

Tongue tissue: Cell growth was observed in 75% of the replicates, representing four individuals. Among the total 45 replicates, 15% and 15% were lost due to fungal and bacterial contamination, respectively. From four individuals, 53% of the replicates reached cell line stage and were cryopreserved. When using Control, ITS, and FGF medium, 53%, 40%, and 60% of the replicates resulted in viable cell lines, respectively.

Toe clip tissue: Cell growth was observed in 35% of the replicates, representing three individuals. Within the total 45 replicates, 42% were lost due to fungi but none due to bacterial contamination. From three individuals, 35% of the replicates reached cell line stage and were cryopreserved. When using Control, ITS, and 2-mercaptoethanol media, 44%, 44%, and 88% of the replicates resulted in viable cell lines, respectively.

Table 3. Growth patterns of *D. melanostictus*. Growth patterns with three different tissue types in various culture media among five individuals. The numbers shown indicate number of replicates performed.

		Lost to infection		Growth patterns		
	Total no. of replicates	Unsuccessful due to fungi	Unsuccessful due to bacteria	No cell growth observed	Culture reached 10–100 cells	Cell lines cryopreserved
Whole limb tissue in media Control/ITS/FGF						
Individual 1	3/3/3	3/3/2	0/0/0	3/3/3	0/0/0	0/0/0
Individual 2	3/3/3	0/0/1	0/0/0	0/1/2	3/2/1	3/2/0
Individual 3	3/3/3	0/0/0	0/0/0	0/1/0	3/2/3	3/2/3
Individual 4	3/3/3	1/1/0	0/0/0	1/0/0	2/3/3	0/1/2
Individual 5	3/3/3	2/0/0	1/0/0	3/2/3	0/1/0	0/1/0
Tongue tissue in media Control/ITS/FGF						
Individual 1	3/3/3	1/0/2	1/3/3	2/3/3	1/0/0	1/0/0
Individual 2	3/3/3	0/0/0	0/0/0	0/0/0	3/3/3	1/0/2
Individual 3	3/3/3	0/0/1	0/0/0	0/0/1	3/3/2	3/2/2
Individual 4	3/3/3	0/0/1	0/0/0	0/0/0	3/3/3	3/3/2
Individual 5	3/3/3	2/0/0	0/0/0	0/1/0	3/2/3	1/1/3
Toe clip tissue in media Control/ITS/2-mercaptoethanol						
Individual 1	3/3/3	1/2/0	0/0/0	3/1/0	0/2/3	0/0/3
Individual 2	3/3/3	0/3/3	0/0/0	2/3/2	1/0/1	0/0/0
Individual 3	3/3/3	1/0/1	0/0/0	3/1/1	0/2/2	0/0/0
Individual 4	3/3/3	0/0/0	0/0/0	2/1/1	1/2/2	1/2/2
Individual 5	3/3/3	3/2/3	0/0/0	3/3/0	0/0/0	3/2/3

2) Cell appearance of whole limb, tongue, and toe clip explant cultures

Cells were categorized based on morphology and are named as such throughout this study.

Whole limb explants (Fig. 1 a–c): Primary cell cultures setup were dominated by fibroblast-like cells (elongated and spindle-shaped) growing in layers, with a few epithelial-like cells growing sporadically in between. After two passages, all epithelial cells were gradually replaced by spindle-shaped fibroblast-like cells. Following cryopreservation, cells displayed similar spindle-shaped fibroblast-like cells as before cryopreservation.

Tongue explants (Fig. 1 d–f): Primary cell cultures displayed a mix of epithelial-like cells (round and cubic cells) and fibroblast-like cells growing in clusters. Epithelial-like cells were distinguished by round/cubic shapes growing in mosaic-like monolayers. After two passsages, round/cubic epithelial-like cells dominated the culture. Following cryopreservation, cells displayed a similar mix of epithelial-like and fibroblast-like cells growing in clusters as seen during the primary cultures.

Toe clip explants (Fig. 1 g–j): Primary cell cultures setup with toe clip tissue were dominated by fibroblast-like cells (elongated and spindle-shaped) growing in layers, with a few epithelial-like cells (round and cubic cells) cells growing sporadically in between. After two passages, epithelial-like cells were gradually replaced by spindle-shaped fibroblast-like cells as seen when working with whole limb tissue. Following cryopreservation, cells displayed similar spindle-shaped fibroblast-like cells as before cryopreservation.

3) Growth patterns of cells from different tissue types

The general growth patterns observed with the three different tissue types were divided into three different periods (Fig. 2): (i) Establishment, from 0–20 h; (ii) First proliferation period, from 20–70 h; (iii) Second proliferation period, from 75–130 h and media change at 72 h. These three time points including media change were selected to characterize each period. As no significant differences in the use of media types were found (Table 3), growth patterns from all three tissue types were chosen based on the number of successful cryopreserved cell lines distributed among the most individuals. Table S1 (Supplementary data) displays CI values of the three selected time points for all three tissue types.

Whole limb tissue (Fig. S1). Growth patterns of cells from whole limb tissue displayed variation among individuals, reaching a CI-level ranging from 0.7–1.8 at time point (i) (Table S4). However, for three out of four individuals, the increase was followed by a decrease in CI-level dominated between the first two time points.

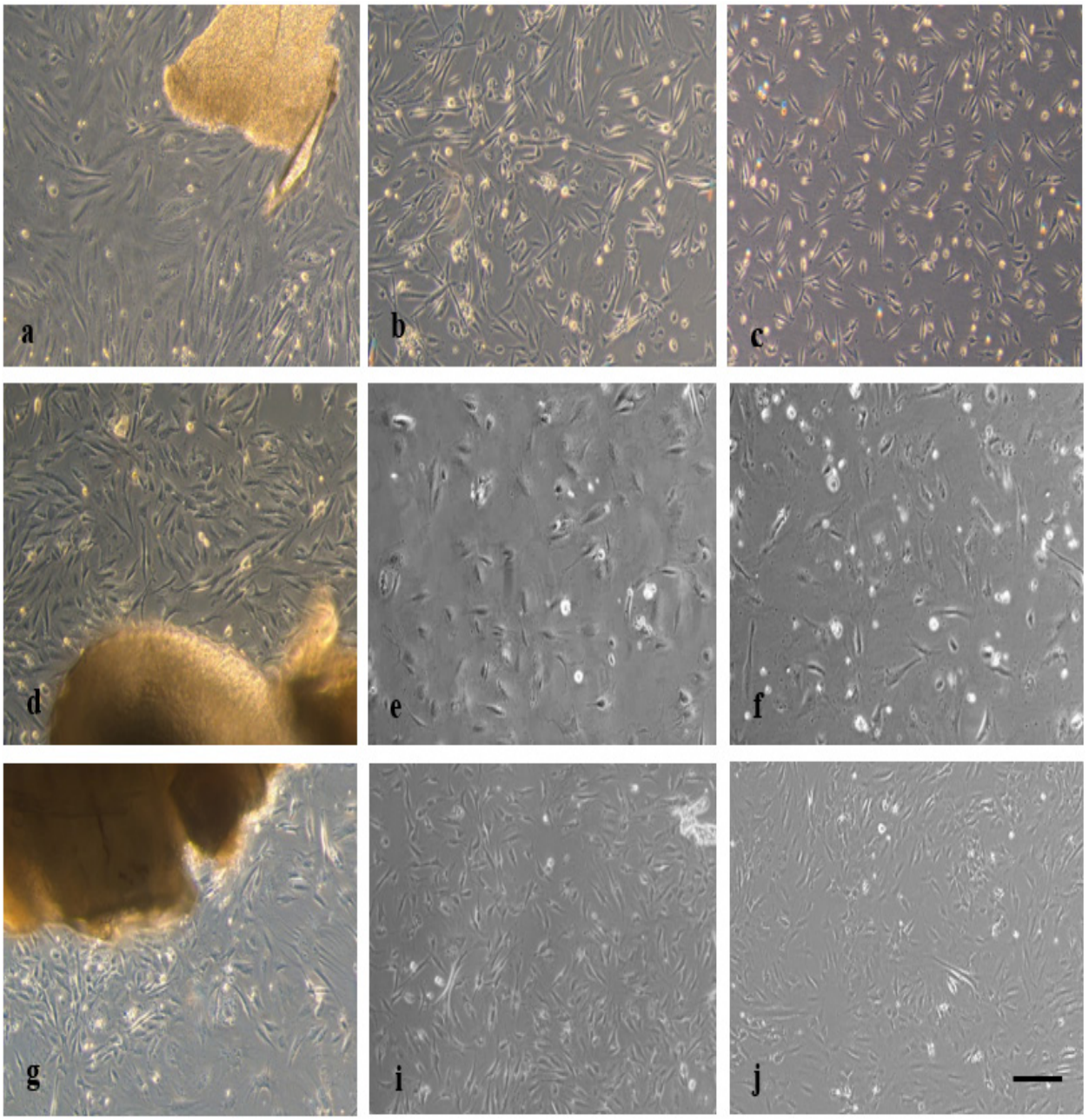

Figure 1. Explant cultures from three different tissue types from *D. melanostictus*. Primary cultures from whole limb explants **(a)** at day 45, **(b)** Passage 2 pre cryopreservation, **(c)** Passage 2 post cryopreservation. Primary cultures from tongue explants **(d)** at day 45, **(e)** Passage 2 pre cryopreservation, **(f)** Passage 2 post cryopreservation. Primary cultures from toe clip explants **(g)** at day 45, **(i)** Passage 2 pre cryopreservation, **(j)** Passage 2 post cryopreservation. Reference bar = 200 μM.

Medium change had an effect on all four cell lines, indicated by a drop in CI-level. After media change, all but one individual reestablished their growth patterns until the last time point (iii).

Tongue tissue (Fig. S2). A general growth pattern was seen among cell lines from four different individuals (Table 3), all ranging from a CI of 0.8–2.8 at time point (i) (Table S4). Between the first two time points an increase in CI was observed among all four individuals, where some displayed a higher growth pattern than others (individual 1 and 4). All cell lines displayed a reaction to media change, however, all resumed growth after media change. Between time points (ii) and (iii), individuals 1, 2, and 4 reached a maximum cell outgrowth CI ranging from 2.9–6.0. A decrease in CI was seen after reaching the maximum cell outgrowth for all three individuals.

Toe clip tissue (Fig. S3). Cell lines set up from toe clip tissue were established for three different individuals (Table 3), and a similar general growth pattern was observed for all three individuals. However, the CI-level differed between all three individuals ranging over a CI of 1.2–2.3 at time point (i) (Table S1 Supplementary

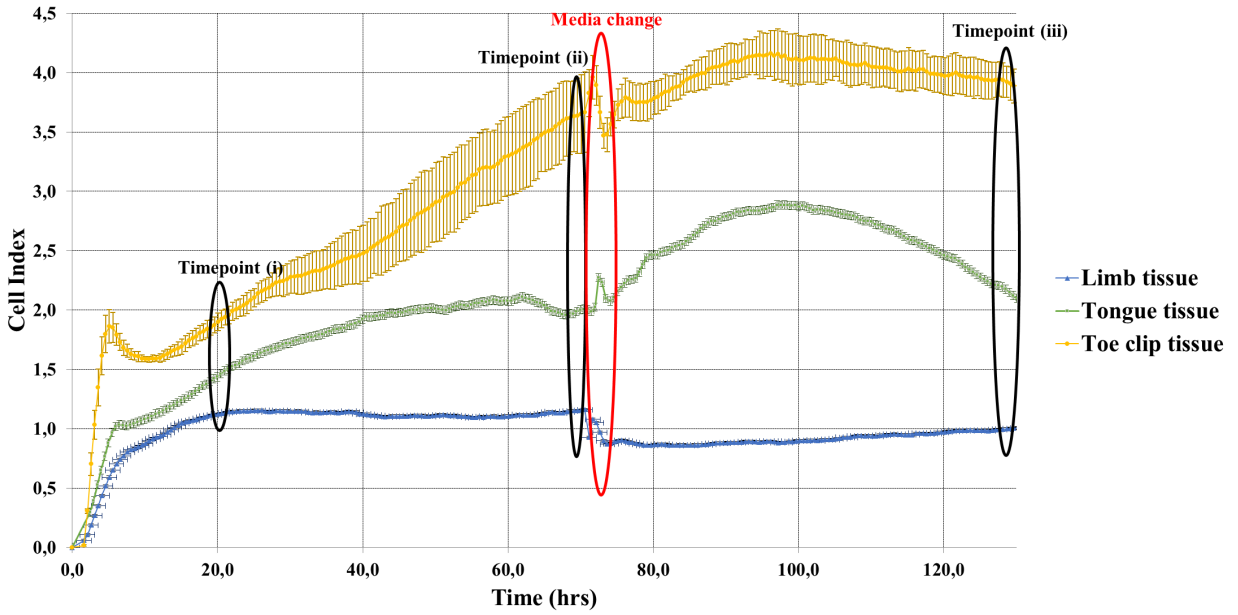

Figure 2. Model displaying the general growth pattern observed using one media type for the three different tissue types (whole limb, tongue, and toe clip tissue), at time points (i), (ii), and (iii)(illustrated in blue circles) including media change (illustrated in red circle).

Data). Between the first two time points individual 1 and 2 both displayed growth patterns reaching a CI-level ranging between 3.8–7.5. All cell lines displayed a reaction to media change, however, all resumed growth after media change. Between time points (ii) and (iii) individuals 1 and 2 reached a maximum cell outgrowth ranging between 4.2–7.5. After reaching the maximum cell outgrowth, a decrease was seen in both individuals, whereas individual 3 displayed a low but continuous increase throughout the study.

4) Variations between individuals using one tissue type

No significant differences were seen between the use of Control, ITS, and FGF media on whole limb tissue. Therefore, the same dataset was used to illustrate the variations seen in growth patterns among the four different individuals including three replicates per individual (Fig. 3). Variations in growth patterns were observed between the four individuals, while a consistent growth pattern was observed between replicates within each individual. At time point (i), individual 1 reached a CI ranging from 1.4–2.2; however, instead of cells spreading and proliferating, a continual loss of adhesion was observed (Table 4). The same tendencies were observed in individual 4. At time point (i), CIs ranged between 0.7–1.2 and 1.1–1.3 in individual 2 and 3, respectively. Between the first two time points a continuous growth pattern was observed. Individual 3 displayed a decrease in CI (due to loss of adhesion) before displaying any growth. Medium change caused changes in all cell lines. However, individual 2, individual 3 and one replicate from individual 1 resumed growth after media change, whereas a continuous decrease was seen for individual 4 and two replicates of individual 1 throughout the study.

Discussion

The use of different tissues when setting up amphibian cell lines

Various tissue types have shown promising results in terms of establishing amphibian cell lines (Houck et al.

Table 4. Cell index (CI) obtained for all individuals at three different time points. Statistical significance was measured between individuals at time points (i), (ii), and (iii) using the xCELLigence® system. Values are means ± SE. Values of $p < 0.05$ are considered to indicate statistically significant differences between CI at different time points.

Time points	Individual 1 ($\mu \pm SE$)	Individual 2 ($\mu \pm SE$)	Individual 3 ($\mu \pm SE$)	Individual 4 ($\mu \pm SE$)	Oneway ANOVA
20 h	1.46 ± 0.2	0.93 ± 0.1	0.94 ± 0.0	1.56 ± 0.2	$F_{3,8}$, $p = 0.0870$
70 h	0.92 ± 0.2	1.08 ± 0.1	0.87 ± 0.0	1.41 ± 0.1	$F_{3,8}$, $p = 0.0807$
130 h	0.58 ± 0.2	1.34 ± 0.2	0.88 ± 0.1	0.63 ± 0.1	$F_{3,8}$, $p = 0.0364$

* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$

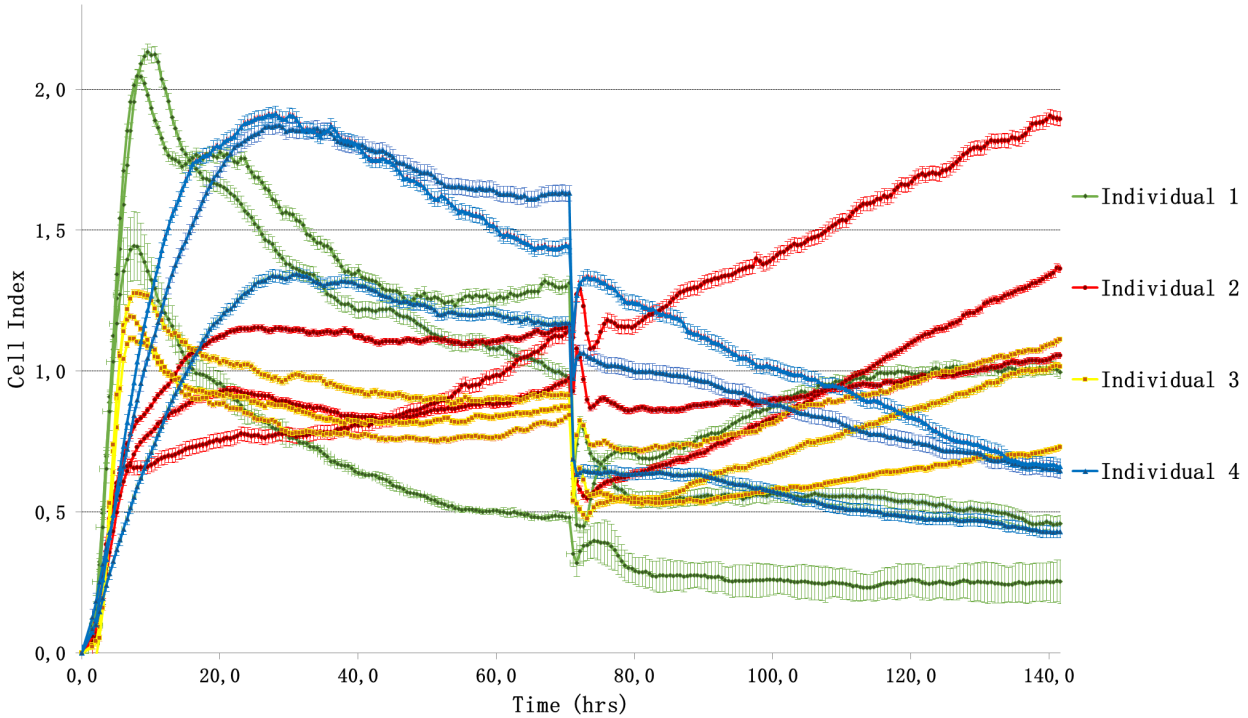

Figure 3. Variation in growth patterns among four different individuals of *D. melanostrictus* using whole limb tissue. Media change was performed after 72 h. Curves are color-coded for each individual displaying three replicates per individual including standard deviations.

2017; Zimkus et al. 2018; Strand et al. 2021). No direct measurements of tissue types and their growth conditions have previously been examined. However, studies focusing on explant vs. enzyme digestion, temperature, media type, supplements, and antibiotics have tested these parameters to varying degrees (see Strand et al. 2021 for an overview of published studies).

Here, we focused on testing growth patterns of amphibian cells lines from three different tissue types: whole limb, tongue, and toe clip. When compared to the two other tissue types (Table 3), tongue tissue was more successful in terms of establishing cell lines. Initial cell growth was observed in 77% of the replicates as compared to 51% and 35% in whole limb and toe clip tissue, respectively. Also, when comparing the number of established and cryopreserved cell lines, 55% of the replicates from tongue tissue were established, versus 38% (whole limb) and 35% (toe clip). In terms of contamination, no considerable differences were seen among the three different tissue types. Cell lines from toe clip have previously been successfully established by Mollard (2018) and, due to the possibilities of sampling non-invasively, this is a very important tissue type to optimize culture protocols from to ensure a high success rate. We suggest further studies focusing on optimizing cell culture protocols using toe clips or toe webbing to improve the success rate when using this specific tissue type.

We chose to work with 100% Cellgro Minimum Essential Medium (MEM) Alpha 1 X (Fisher Scientific), which had already proven successful in establishing amphibian cell lines (Houck et al. 2017; Strand et al. 2021). Moreover, we chose to study the effect of three different supplements (FGF, ITS, and 2-mercaptoethanol; Table 2). The choices of supplements were based on the advantages of each supplement; FGF sustains the cellular proliferation and differentiation of fibroblast cells; ITS contains insulin, transferrin, and selenium, which are essential for growth, glucose and amino acid uptake, intercellular transport, transportation of iron, reducing oxygen radicals and peroxide and function as co-factor for other proteins; 2-mercaptoethanol is used to stimulate proliferation and functions as a reducing agent to prevent toxic levels of oxygen radicals (Davis 2011; Click 2014; Freshney 2016; Houck et al. 2017; Verma et al. 2020; Strand et al. 2021). When comparing results on growth patterns for whole limb and tongue tissue (Table 3), 13%, 13%, and 11% of the replicates resulted in cell lines using Control, ITS, and FGF media, respectively. When using tongue tissue, 20%, 13%, and 20% of the replicates resulted in cell lines using Control, ITS, and FGF media, respectively. When comparing data from these two specific tissue types and the use of different media, no considerable pattern was seen. However, when testing Control, ITS, and 2-mercaptoethanol media with toe clip tissue we saw a positive tendency when using 2-mercaptoethanol media. Similar positive results were published by Strand et al. (2021), showing that mercaptoethanol can have a positive effect on growth initiation when using cryopreserved tissue explants.

Differences in growth patterns between different tissue types

The xCELLigence® system was used to provide real-time measurements of the cell growth, and this system has been widely used in studies with humans, mice, and rats (Nad et al. 2010; Urcan et al. 2010; Rakers et al. 2014; Kho et al. 2015) but only once before for amphibians by the same research group (Strand et al. 2021). In general, a lower CI was seen among cell lines of both whole limb and tongue tissue. For whole limb tissue, the maximum CI observed in this study ranged between 1.2–2.2, which is defined as weak (Kho et al. 2015). For tongue tissue, the maximum CI ranged between 1.4–6.0 (weak to moderate), and for toe clip tissue from 1.5–7.5 (weak to moderate). Strand et al. (2021) found the maximum CI for *Triturus cristatus* to range between 6.5–7.2 (moderate to strong), indicating the need to optimize seeding density when working with both a new species as well as different tissue types. Figure S1 displays the variation in growth patterns when using whole limb tissue to establish cell lines, and only a few similar growth patterns among the four individuals were observed, indicating heterogenic cell type combinations in each of the individuals. Compared with tongue tissue (Fig. S2), samples were more homogeneous in terms of growth patterns, and the variations observed between individuals were reduced when using tongue tissue. Similar observations were seen when using toe clip tissue for setting up cell lines, however, a variation in CI-level was observed among the three individuals (Fig. S3). Different cell types produce different CI curves, but studies analyzing fibroblast-like cell lines from fish, human, rat, and mice have found similar growth patterns using fibroblast-like fish and rat cell lines (Rakers et al. 2011; Rakers et al. 2014). Here a drop in CI was also observed after the adhesion phase and before entering the plateau or growth phase. This pattern is like the pattern observed when using cell lines established from whole limb tissue, which was dominated by fibroblast-like cells. However, because this study was using cell lines of multiple cell types, the variations in their fingerprints cannot be directly compared, but similar patterns were detected pending the dominant cell type.

Differences in growth patterns among individuals

Based on the information from (Zimkus et al. 2018), successful conditions and methods used for some species failed to work on others, even within the same genus, we decided to test the differences in growth patterns among four individuals within a species (Fig. 3), using three cell line replicates within each individual. In general, the growth patterns among the replicates within each of the individuals displayed no significant variations, but instead a consistent growth pattern was seen. As displayed in Fig. 3a variations in growth patterns between the four individuals were detected, as no cell proliferation was observed in individuals 1 and 4 after initial seeding except for one replicate, whereas individuals 2 and 3 displayed proliferating growth patterns before and after media change. Even though it was possible to establish cell lines for all four individuals, the growth patterns of the cell lines showed differences when thawed, indicating that some individuals are more successful with long-term cultures than others. This highlights the necessity of consistent quality checks of all established cell lines to ensure viability and continuous proliferation patterns after cryopreservation.

In summary, the results from this study provide new knowledge concerning growth patterns of three different tissue types used for establishing amphibian cell lines. The usability of all three tissue types were demonstrated by successful establishment of cell lines, however, the success rate was found to be higher and the growth patterns to be more consistent when using tongue tissue. No considerable preference according to media was found among tissues from whole limb and tongue, however, toe clip tissue displayed positive tendencies when using media D. Growth patterns were found to be consistent among the three replicates within each individual, however, variations were found when testing the individuals against each other. These results provide basic knowledge in terms of choosing tissue type and media preferences as well as displaying differences in growth patterns found among individuals. This information will be useful when designing future studies on amphibian cell lines where the importance of successful storage of viable genetic material have never been more important than during a global amphibian crisis.

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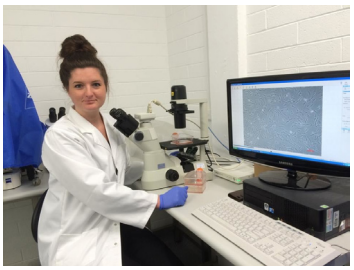

Julie Strand's main research interests are applied cell biology, cytogenetics, and conservation biology. Her research focuses especially on biobanking as a conservation and management tool. She specializes in establishing viable cell lines from amphibians and reptiles, and is currently working on adding comparative cytogenetics as a skill, thereby being able to combine biobanking and comparative cytogenetic information in her future work within conservation management.

Henrik Callesen is professor in reproductive biology and technology in farm animals at Aarhus University in Denmark. His research interests include the biological mechanisms behind various reproductive technologies as well as their consequences on gametes, fetus, and offspring when used in different species, primarily cattle and pig.

Cino Pertoldi's research focuses on empirical conservation and evolutionary genetics of animals, but also includes conceptual and theoretical studies in the interface between genetics, ecology, and evolution. He has merged current efforts in evolutionary and ecological genetics, complementing molecular genomics and macroecology in order to understand how genetic measures can indicate causal processes.

Stig Purup's main interests are applied cell biology, bioactive components, and lactation physiology. His research includes investigation of bioactive components in food and feedstuff and their importance for the nutritional and health beneficial effect. A specific research area of interest is bioactive components in natural mixtures such as milk, blood, and plant- and tissue extracts. The research also includes the lactation physiology associated with regulation, synthesis, and secretion of bioactive components from the mammary gland into the milk. His team is proficient in isolation of primary mammary epithelial cells and establishment of cell models for studying physiological development of the bovine mammary gland and lactogenesis.

Supplementary Data

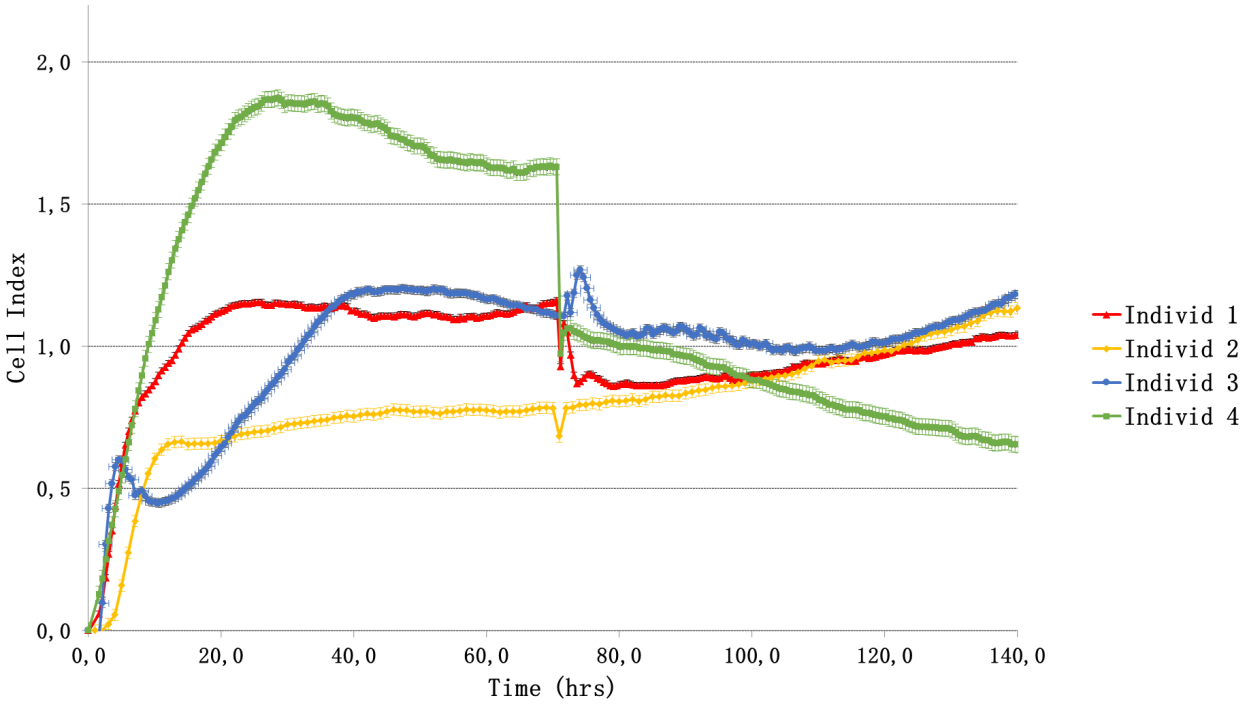

Figure S1. Variation in growth patterns of limb tissue among four different individuals of *D. melanostictus*. Curves represent the mean cell index value from three replicates including standard deviations.

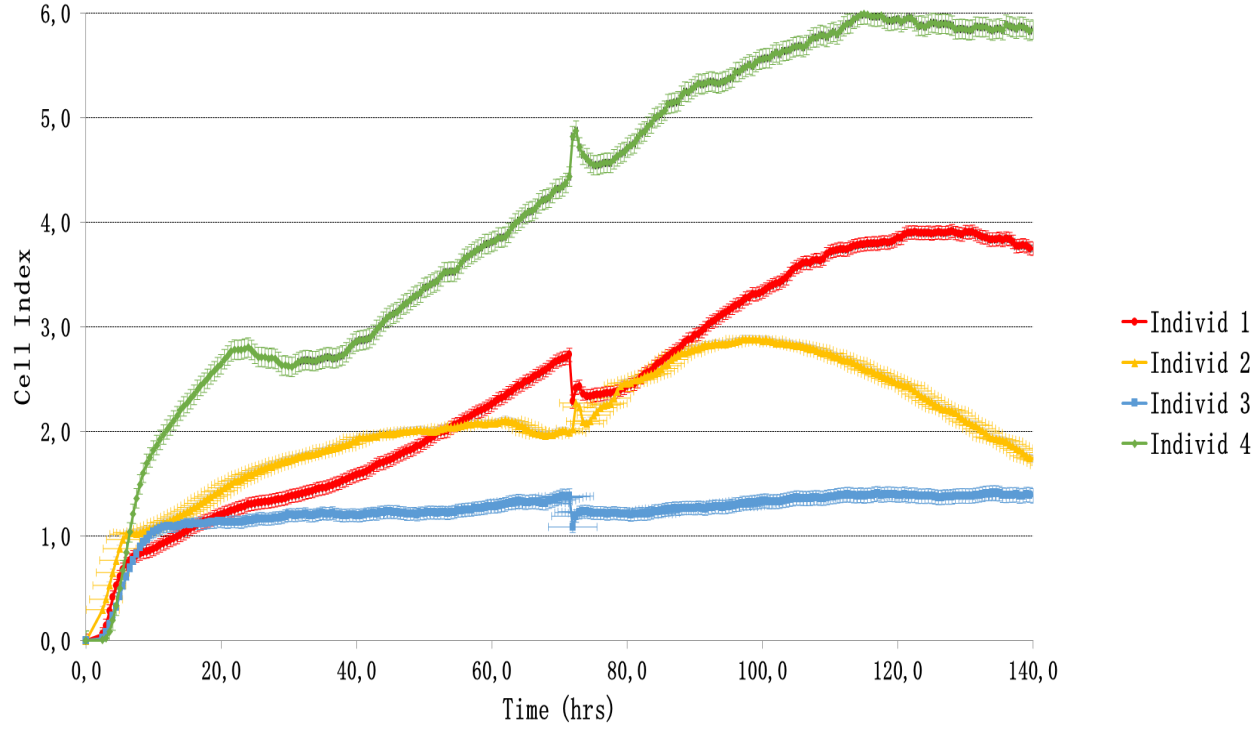

Figure S2. Variation in growth patterns of tongue tissue among four different individuals of *D. melanostictus*. Curves represent the mean cell index value from three replicates including standard deviations.

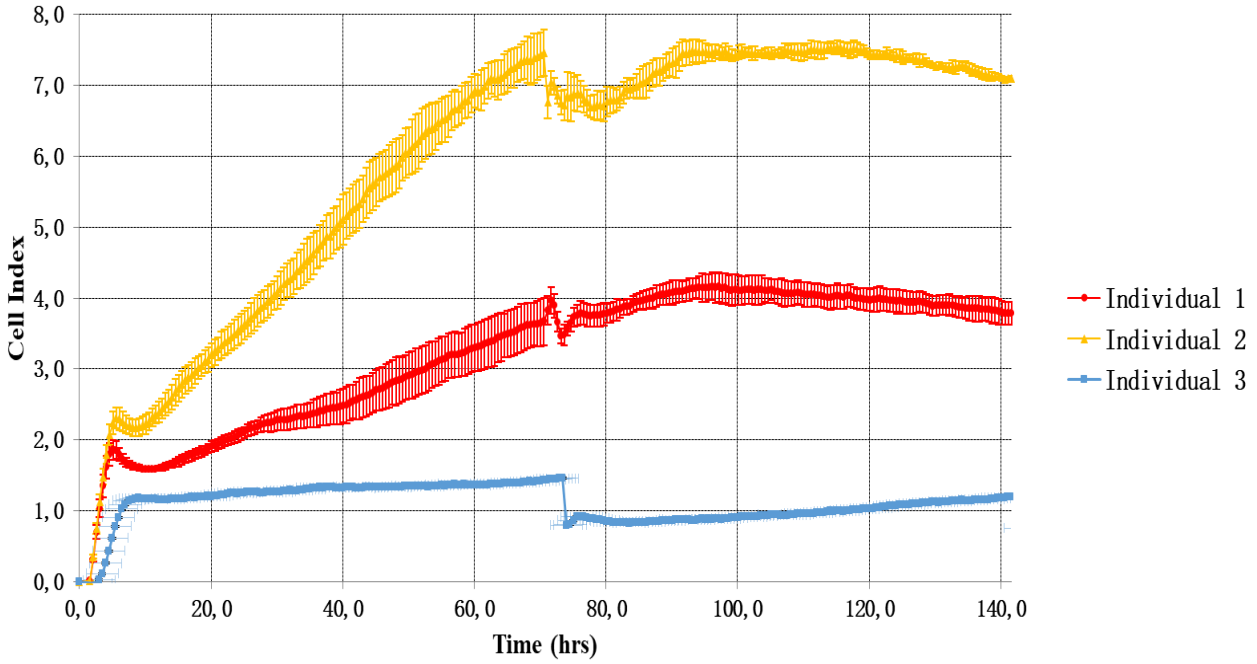

Figure S3. Variation in growth patterns of toeclip tissue among three different individuals of *D. melanostictus*. Curves represent the mean cell index value from three replicates including standard deviations.

Table S1. Cell index (CI) obtained for all individuals at three different time points. Statistical significance was measured between individuals at time points (i), (ii), and (iii) using the xCELLigence® system. Values are means ± SE. Values of $p < 0.05$ are considered to indicate statistically significant differences between CI at different time points.

Time point/ Limb	**Individual 1 ($\mu \pm SE$)**	**Individual 2 ($\mu \pm SE$)**	**Individual 3 ($\mu \pm SE$)**	**Individual 4 ($\mu \pm SE$)**	**One-way ANOVA**	**Tukey's test**
20 h	1.12 ± 0.1	0.62 ± 0.1	0.65 ± 0.0	1.71 ± 0.1	$F_{3,8}$, $p = 0.00017$	(A>C**, A<D**), (B<D***), (C<D***)
70 h	1.15 ± 0.1	0.87 ± 0.1	1.11 ± 0.0	1.63 ± 0.1	$F_{3,8}$, $p = 0.00080$	(A<D*), (B<D***), (C<D**)
130 h	1.01 ± 0.1	1.00 ± 0.1	1.09 ± 0.0	0.71 ± 0.1	$F_{3,8}$, $p = 0.05157$	(C>D*)
Time point/ Tongue	**Individual 1 ($\mu \pm SE$)**	**Individual 2 ($\mu \pm SE$)**	**Individual 3 ($\mu \pm SE$)**	**Individual 4 ($\mu \pm SE$)**	**One-way ANOVA**	**Tukey's test**
20 h	1.23 ± 0.1	1.49 ± 0.1	1.13 ± 0.1	2,72 ± 0.1	$F_{4,10}$, $p = 0.00043$	(A<D***), (B>C*, B<D***), (C<D***)
70 h	2.70 ± 0.1	2.00 ± 0.2	1.37 ± 0.1	4.37 ± 0.3	$F_{4,10}$, $p = 0.00005$	(A>C**, (A<D***), (B<D***), (C<D***)
130 h	3,91 ± 0.1	2.06 ± 0.1	1.38 ± 0.2	5.84 ± 0.5	$F_{4,10}$, $p = 0.00007$	(A>B**, (C***, D**),(B<D***), (C<D***)
Time point/ Toe clip	**Individual 1 ($\mu \pm SE$)**	**Individual 2 ($\mu \pm SE$)**	**Individual 3 ($\mu \pm SE$)**		**One-way ANOVA**	**Tukey's test**
20 h	1.90 ± 0.0	3.19 ± 0.1	1.20 ± 0.0		$F_{2,6}$, $p = 0.00348$	(A<B***, (A>C***),(B>C***)
70 h	3.65 ± 0.1	7.43 ± 0.1	1.42 ± 0.0		$F_{2,6}$, $p = 0.00363$	(A<B***, (A>C***),(B>C***)
130 h	3.89 ± 0.0	7.26 ± 0.0	1.12 ± 0.1		$F_{2,6}$, $p = 0.00369$	(A<B***, (A>C***),(B>C***)

* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$