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Review

Essential features of *in vitro* fish cell culture: an overview

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ABSTRACT. The reports of fish cell cultivation date back to the early 20th century, and since then, it has become a tool of great importance for different areas of ichthyology, such as immunology, toxicology, and biotechnology. The cell cultivation methodology can vary according to the species and cell type, requiring knowledge of the specificities and characteristics of the target cell for them to be efficiently cultured. In this review, we address the main characteristics of the *in vitro* culture of fish cells, presenting their purposes, uses, main parameters, and different characteristics necessary for the culture environment. Finally, we present the experiences in Brazil using cell culture techniques.

Keywords: fish; *in vitro* assays; cell culture; neotropical fish species, Brazil

INTRODUCTION

Cell culture is a set of techniques where cells of interest are isolated and cultivated *in vitro*. Through cell culture, it is possible to study specific functions, interactions, biochemical components, and physiology of a given cell line (Oyeleye et al. 2016). Over the decades, cell culture methods have been constantly improved, and assays using *in vitro* cultured cells have become indispensable for several areas of science, such as medicine and agriculture. Their use is strongly encouraged for ethical, scientific, and economic reasons (Bols et al. 2005, Castaño & Gómez-Lechón 2005, Schirmer 2006, Taju et al. 2014).

The reports involving cell cultivation of fish date back to the early 20th century (Wolf & Quimby 1969); since then, the use of fish cells as an experimental model has gained increasing attention due to the high genetic plasticity of these organisms, the great diversity of species and the fact that they live in the aquatic environment, where, evolutionarily, they have adapted to the exposure to the most diverse environmental pressures (Cossins & Crawford 2005).

In Brazil, studies using cell culture techniques are still scarce; however, in the last years, there has been an increase in their numbers, either using native fish species such as *Hoplias malabaricus* (Ribas et al. 2014, 2016), *Astyanax lacustris* (former *A. altiparanae*) (Paim et al. 2018, Levy-Pereira et al. 2023), and *Piaractus mesopotamicus* (Duran et al. 2019); or Nile tilapia (*Oreochromis niloticus*) (Rocha et al. 2009), which although an allochthonous species, corresponds to more than 60% of the Brazilian aquaculture production (PeixeBr 2023). Moreover, these studies investigated different research fields, such as cytogenetics, immunology, toxicology, and nutrition physiology.

In this comprehensive review, we aim to explore crucial considerations for cultivating fish cells. We aim to update key information about the most commonly utilized cell types while briefly introducing noteworthy Brazilian experiences in this domain.

The purpose of cell culture

In vitro cell cultivation aims to maintain the desired cells outside their natural organism in a healthy and

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functional state (Oyeleye et al. 2016). This allows these cells to be used for studies in various areas of science, such as virology, biotechnology, cytology, toxicology, and molecular biology (Sobhana et al. 2009, Short & Wagey 2013, Oyeleye et al. 2016).

Instead of whole fish bioassays, cell culture has several advantages when compared to *in vivo* experimentation, such as enabling assay species threatened with extinction to perform studies using very large or very small species to using the cells from the same fish for treated and control groups decreasing the intraassay genetic variation; and to study the effect of toxic substances without the need to euthanize a large number of fish (Amemiya et al. 1984). Moreover, with the creation of cell banks, the need for fish killing can be even more reduced, contributing to ensuring that ethical principles are followed during the execution of the analyses (Sneddon et al. 2017, Paim et al. 2018). Additionally, assays using cell culture are carried out under strictly controlled environmental conditions, such as pH, temperature, salinity, osmolality, and amino acids concentration, providing a better understanding of some biological phenomena, increasing the reliability of the assays, and minimizing waste production (Takhar & Mahant 2011).

Initial considerations for the culture of fish cells

Lakra et al. (2011) and Rubio et al. (2019) elegantly elucidated the fundamentals of fish cell culture. Nevertheless, certain key distinctions must be emphasized, enabling a clear differentiation mainly between cell cultivation techniques in fish and mammals. Additionally, this delineation can save valuable time, as some aspects remain consistent. At the same time, others obviate the necessity for novel approaches when adapting cell culture protocols from other animal models to fish.

Firstly, it is essential to consider several key factors to understand their traits better when studying fish. Firstly, the fish family encompasses over 34,000 species, more than all other vertebrates combined (Weitzman & Parenti 1999). This wide variety has led to extended evolutionary histories for some fish groups compared to most mammals (Volff 2005), resulting in significant differences in how cells respond among different fish species.

The availability of immortalized fish cell lines is another crucial point to note. Cell immortalization has been a significant advancement in cell culture, enabling the establishment of permanent cell lines without the need for repeated primary isolations. A list of species with available cell lines can be found in Table 1. However, fish immortalized cell lineages are relatively scarce compared to the many fish species, particularly when compared to mammals, and none of these have been derived from Brazilian or Neotropical fish species.

Additionally, when performing cell subculture, which involves working with primary cells after numerous passages or immortalized lines, there is often less consistency in cell characteristics in terms of both morphology and function compared to freshly isolated primary cells (Stacey 2006, Rymuszka et al. 2021). As a result, researchers often find themselves in a situation where they must initiate primary cell cultures for significant fish species in their region. Furthermore, these species may lack comprehensive literature-based protocols or accessible immortalized cell lines.

A shared characteristic between some fish cell types and mammals is their ability to stick to plastic or glass surfaces. This feature allows the formation of adherent cell layers in culture vessels, making it easier to assess cell growth rates and evaluate the impact of pathogens and foreign substances (Kim et al. 2014). However, it is important to note that certain cell types, such as stem cells, hematopoietic cells, and transformed tumor cells, are known for not adhering so well and require suspension culture techniques for their growth (Molinaro et al. 2013, Khalili & Ahmad 2015, Merten 2015).

Essential parameters for cell culture according to cell types

Several important parameters must be considered to succeed in cell culture, particularly for fish cells. These parameters, including cell origin, temperature, medium type, and media enrichment, vary significantly depending on the fish species from which the cells were isolated and the specific cell type to be cultured. In the upcoming discussion, we will highlight the key parameters that need to be considered and analyze their significance concerning the four most frequently employed cell types in fish experiments.

Culture medium

As well as for bacteria, the culture media plays a crucial role in the cell culture, simulating the natural tissue environment by providing suitable nutrients, pH, and osmolarity for cell survival and multiplication (Freshney 2010). In the case of mammalian and fish cells, the composition of the medium depends on the specific cell type being cultured, as well as the species from which the cells are isolated, resulting in different requirements for amino acids, sugars, and antibiotics (Yao & Asayama 2017, Shiomi et al. 2020). Also, the media must present a capacity to support the cells'

Table 1. Different species with established cell lines.

Species	Cell type	Reference
Danio rerio	Epithelial and fibroblast	ATCC (2021)
Oncorhynchus mykiss	Epithelial and fibroblast	ATCC (2021)
Lepomis macrochirus	Fibroblast	ATCC (2021), ECACC (2021)
Clarias batrachus	Pleomorphic	ATCC (2021)
Sparus auratus	Fibroblast and epithelial	ECACC (2021)
O. mykiss	Fibroblast and epithelial	ECACC (2021)
Cyprinus carpio	Squamous, epithelial and fibroblast	ECACC (2021)
O. kisutch	Fibroblast	ECACC (2021)
O. tshawytscha	Fibroblast	ECACC (2021)
O. nerka	Epithelial	ECACC (2021)
Ictalurus punctatus	Fibroblast	ECACC (2021)
Esox lucius	Fibroblast	ECACC (2021)

metabolic waste during the culture period while maintaining its chemical characteristics, such as pH, so as not to harm the cells. Hence, because of the nutrient depletion and the excess of metabolic waste, it is recommended to change the medium periodically to ensure the renewal of essential nutrients for cell growth (Paim et al. 2018), keeping in mind that the high frequencies of medium change can result in loss of non-adherent cells, what can be a problem in some cases. A practical solution to this issue is to use a substrate like a gelatin matrix for cell attachment, as Paim et al. (2018) demonstrated, which minimizes cell losses during medium changes.

Leibovitz L-15 is rich in amino acids and requires no CO_2 buffer (Leibovitz 1963). It has shown successful results in fish cell lines, with over 80% of strains established after 1994 utilizing this medium (Lakra et al. 2011). Other media commonly used for cell cultures, such as Eagle's minimal essential medium (EMEM), are often developed for mammalian cells, which are typically supplemented with Fetal bovine serum (FBS). This medium is widely used for cell culture in various species, including mammals, birds, reptiles, fish, and amphibians (Wolf & Quimby 1969). The Roswell Park Memorial Institute (RPMI-1640) medium is commonly employed for fish immune cell culture. This medium offers a wide range of amino acids, vitamins, salts, and sugars and has been extensively used in fish immune cell culture studies (Moore et al. 1967, Ottinger & Kaattari 1998, Rymuszka et al. 2021). Other media have been used for fish cell culture.

The optimal medium for *in vitro* cell culture depends on the specific cell type. Additionally, it is crucial to consider that media can vary in price depending on the brand and may require importation, resulting in potentially long waiting times that can cause delays in research dependent on their arrival.

Enrichment of mediums

Media used in cell culture to optimize cell development are often supplemented with components that stimulate growth and proliferation, providing the necessary energy for satisfactory cell development (Gstraunthaler 2003). However, it is crucial to consider the significant differences between mammalian and fish cells. For instance, most fish species cells typically require lower incubation temperatures, and marine fish cells often require higher osmolarity (Lakra et al. 2011).

It is important to note that nutrients play a critical role as limiting factors in cellular growth. Depletion of nutrients in the cellular environment can lead to a decline in glucose and amino acid concentrations, resulting in cellular stress (Duboc & Von Stockar 2000).

L-glutamine, glucose, and cysteine are among the most common nutrients cells use as energy sources (Oyeleye et al. 2016), and they are frequently found in commercial formulations of culture media. L-glutamine supports cell growth in cells that require substantial energy and synthesize high amounts of proteins and nucleic acids. It serves as an alternative energy source for cells with rapid division and inefficient use of glucose, promoting cell maturation and modulating substrate metabolism (Cardin et al. 2000, Le Bacquer et al. 2001). Cysteine also plays a vital role in cell growth and metabolism while providing protection against free radicals (Abbas et al. 2015, Khalilzadeh et al. 2015).

Certain components in culture media are indispensable for successful cell culture techniques. For example, FBS promotes cell growth but is also the most expensive option (Wolf & Ahne 1982). However, Rosa et al. (2010) reported positive results by substituting FBS with serum obtained from *Sparus aurata* fish cells, possibly due to taxonomic similarity.

Other substances, such as minerals and hormones, have been described as media enrichment agents, but their usage is highly specific and depends entirely on the purpose of the experiment (Oyeleye et al. 2016).

pH and temperature

The pH of the culture medium is a crucial factor that influences and regulates cell growth. It significantly affects the culture's growth rate and cell density. For optimal growth, most cells require a pH close to 7. The pH level is determined by various factors such as the composition of the medium, the aeration conditions of the culture, the buffers used, and the cellular metabolites (Oyeleye et al. 2016). Phenol red is commonly added to the culture medium to monitor the pH of the cellular environment. The color exhibited by the medium indicates the pH level (Freshney 2005).

Temperature is another vital factor affecting cell growth and has influencing and limiting effects. The ideal temperature range for cell cultures varies depending on the specific cell type and the animal species from which the tissue was derived (Yang & Xiong 2012).

Common cell types for *in vitro* culture and assays

Fibroblast and epithelial cells

Fibroblasts and epithelial cells have gained widespread popularity as essential components in *in vitro* assays due to their remarkable versatility. Fibroblasts stand out for their ease of isolation from various tissues, strong adherence to plastic and glass surfaces, and notable responsiveness to diverse compounds and substances of interest.

In fish research, fibroblasts have been extensively utilized *in vitro* assays, boasting distinct culture attributes, as summarized in Table 2. From a comprehensive literature review, the most commonly used culture media for fish fibroblasts were L-15 and DMEM, reflecting preferences observed in mammalian species. The typical concentration of FBS used for fish fibroblastic/epithelial cells was 10%. However, some studies, such as Abdul-Majeed et al. (2013) for *Channa striatus* and Wang et al. (2013) for *Anabarilius graham*, opted for higher concentrations of 15 and 20% FBS, respectively. Additionally, for *Danio rerio*, Peng et al. (2019) supplemented the culture medium with 2.5% common carp (*Cyprinus carpio*) serum and the standard 10% Fetal calf serum (FCS). These variations in culture conditions underscore the adaptability of fibroblasts for specific research needs in fish studies.

By leveraging fibroblasts' inherent qualities and their response to diverse culture conditions, *in vitro* assays in fish research have witnessed remarkable advancements, offering invaluable insights into various physiological and pharmacological phenomena.

The choice of additives for cell culture varied significantly across studies, driven by the specific objectives of each investigation. Our focus centered on additives that are crucial in cell maintenance and metabolism. Penicillin and streptomycin were frequently employed among the commonly used additives to prevent accidental contamination. Furthermore, antifungals were included as additives in two studies utilizing fibroblasts as a model organism. Abdul-Majeed et al. (2013) used fungizone for *C. striatus*, while Fu et al. (2015) employed amphotericin B for *Siniperca chuatsi*.

Other additives that demonstrated potential in promoting cell adherence and growth included epidermal growth factor and basic fibroblast growth factor, which were utilized in the case of *S. chuatsi* (Fu et al. 2015). For *D. rerio*, Peng et al. (2019) supplemented the culture media with 2-mercaptoethanol, sodium pyruvate, and non-essential amino acids.

Throughout the literature consulted, fibroblasts were isolated from various sources, including the head kidney (Abdul-Majeedet al. 2013), brain (Fu et al. 2015), heart, spleen, testis, ovary (Vo et al. 2015), and embryos (Peng et al. 2019).

The culture temperature showed little variation across the assays, with a consensus around 28°C. However, *O. mykiss* and *Sander vitreus* were incubated at 16°C in the study by Vo et al. (2015). This study revealed the widespread distribution of fibroblasts in fish organisms, as fibroblastic, endothelial, or epithelial cells were isolated from seven different organs of two fish species.

Among the studies reviewed, the only Brazilian study involving fibroblasts focused on isolating them from *A. lacustris* muscle. This study aimed to develop a new technique for obtaining mitotic chromosomes for cytogenetic assays. Table 2 shows no significant differences between the protocols for fibroblast culture of *A. lacustris* and those for other species. The culture

Table 2. The cultivation conditions of fibroblasts and epithelial cells. FCS: fetal calf serum.

Species	Medium	FCS	Culture additives	Origin	Cell culture temperature	Cell type	Reference
Channa striatus	L-15	15%	Penicillin, 100 IU mL^{-1}, streptomycin, 100 µg mL^{-1}) and Fungizone (2.5 µg m L^{-1})	Head kidney	28°C	Fibroblastic	Abdul-Majeed et al. (2013)
Anabarilius grahami	DMEM/F12	20%	100 IU mL^{-1} of penicillin, and 100 ug mL^{-1} of strepto-mycin	Caudal fin	28°C	Fibroblastic	Wang et al. (2013)
Siniperca chuatsi	L-15	20% and 10%	100 IU mL^{-1} penicillin, 100 µg mL^{-1} streptomycin and 0.25 µg mL^{-1} amphotericin B, 10 ng mL^{-1} epidermal growth factor and 10 ng mL^{-1} basic fibroblast growth factor	brain	28°C	Fibroblastic	Fu et al. (2015)
Oncorhynchus mykiss *Sander vitreus*	L-15	10%	200 U mL^{-1} penicillin and 200 µg mL^{-1} streptomycin	Anal fin Heart Spleen Skin Testis Ovary Ovarian fluid Milt	26°C	Epithelial Epithelial/endothelial/ fibroblastic Epithelial/fibroblastic Fibroblastic Epithelial Fibroblastic EpithelialFibroblastic	Vo et al. (2015)
Astyanax lacustris	DMEM	10%		Muscle	28°C	Fibroblastic	Paim et al. (2018)
Danio rerio	DMEM	10%	100 U mL^{-1} penicillin, 100 µg mL^{-1} streptomycin, 2.5% fish serum (common carp), 0.1% 2-mercaptoethanol, 1 mM so-dium pyruvate, and 1 mM non-essential amino acids	Caudal fin and embryos	28°C	Fibroblastic	Peng et al. (2019)

medium used was DMEM, supplemented with 10% FCS, and no additional media additives were employed. The incubation temperature was set at 28°C.

Leukocytes

The immune system plays a crucial role in defending the organism against pathogens through generic and specific responses mediated by a diverse range of cells, including macrophages, neutrophils, and lymphocytes. These cells can be cultivated individually or in co-cultures (Evensen 2020), allowing for the observation of various immunological phenomena, such as phagocytosis, cell migration, production of immune substances responsible for pathogen killing or cell communication, and the expression of immunological genes (Forlenza et al. 2011). Immunological assays are typically conducted using purified cell suspensions or monolayers (Li et al. 2013, Espinosa et al. 2018).

Researchers commonly employ solutions containing silica microspheres with higher densities to isolate leukocytes than the culture media. Commercially known as Percoll or Ficoll solutions, these solutions serve as essential components in the isolation process. Table 3 provides a comprehensive overview of the various concentrations utilized for both Percoll and Ficoll solutions.

In most experiments, multiple Percoll solutions with varying concentrations are employed to enhance the purity of the resulting cell solution. This approach is particularly effective in obtaining high-quality monocyte/ macrophage solutions. Regarding culture media, RPMI, followed by L-15, was the most frequently used for leukocytes (Table 3). RPMI is widely recognized as the most suitable medium, but L-15 can be considered a viable alternative. Additionally, some studies observed DMEM for *Ctenopharyngodon idellus* (Zheng et al. 2019, Rymuszka et al. 2021) and EMEM for *C. carpio* (Li et al. 2020).

The concentration of FCS varied more extensively for leukocytes compared to fibroblasts, ranging from 0.02% for *Scophthalmus maximus* (Zhang et al. 2020) to 20% for *Labeo rohita* (Awasthi et al. 2013). However, in most consulted studies, FCS concentrations between 5 and 20% were preferred.

The primary source of leukocytes is the head kidney, the main hematopoietic organ in fish, analogous to the bone marrow in mammals. However, leukocytes have also been isolated from the peritoneal

Table 3. Key features of *in vitro* cultivation of leukocytes. FCS: fetal calf serum. *Numbers separated by one or more slashes indicate that a gradient of Percoll was used. **Medium enriched with 5% of heat-inactivated carp serum. ***Medium enriched with 1,5% of heat-inactivated carp serum.

Species	Percoll or Ficoll concentration (%)*	Medium	FCS	Origin	Cellular type	Cell culture temperature	Reference
Sparus aurata, *Oncorhynchus mykiss* and *Dicentrarchus labrax*	34/51	L-15	0.10%	Head kidney	Macrophage	13°C	Skarmeta et al. (1995)
Cyprinus carpio	51	L-15	10%**	Head kidney	Macrophage	27°C	Joerink et al. (2006)
Clarias batrachus	34/51	RPMI-1640	10%	Head kidney	Macrophage	30°C	Banerjee et al. (2012)
Labeo rohita		L-15	20%	Peritoneal cavity	Macrophage	30°C	Awasthi et al. (2013)
Hoplias malabaricus	40/60	L-15	2%	Head kidney	Monocytes	20°C - 22°C	Ribas et al. (2016)
Sparus aurata and *Dicentrarchus labrax*	34/51	RPMI-1640	5%	Head kidney	Leukocytes	22°C	Espinosa et al. (2018)
Acipenser persicous and *Rutilus frisii kutum*	51/54	L-15	1%	Head kidney	Macrophage	23°C	Rastgar et al. (2018)
Tatoaba macdonaldi	51	RPMI-1640	5%	Head kidney and spleen	Leukocytes	23°C	Reyes-Becerril et al. (2018)
Ctenopharyngodon idellus	34/51	DMEM	5%	Head kidney	Leukocytes	27°C	Zheng et al. (2019)
Larimichthys crocea	31/45	DMEM/F12	5%	Non specified	Macrophage	26°C	Li et al. (2020)
Cyprinus carpio carpio	51.7	RPMI-1640	***	Head kidney	Macrophage	27°C	Wentzel et al. (2020)
Scophthalmus maximus	35/51	L-15	0.02	Head kidney	Macrophage	24°C	Zhang et al. (2020)
Cyprinus carpio	46.2/53.8/61.5	EMEM and cRPMI-1640*	10% (for EMEM only)	Carp leukocyte cell line (CLC) and head kidney	Leukocytes	26°C and 22°C	Rymuszka et al. (2021)
Astyanax lacustris	42/60	RPMI-1640	10%	Head kidney	Leukocytes	25°C	Levy-Pereira et al. (2023)

cavity (Awasthi et al. 2013), and spleen (Reyes-Becerril et al. 2018). The most commonly isolated and cultured leukocyte subset was from the monocyte/ macrophage lineage, given their crucial role as the first line of defense against pathogens in fish. Macrophages interact extensively with various cells of the innate and adaptive immune systems and exhibit phagocytic abilities, strong adherence to surfaces, and a longer lifespan than other leukocyte types.

In addition to the FCS concentration, leukocyte incubation temperature varied considerably, ranging from 13 to 30°C. This variation often reflects the characteristics of each fish species, with lower temperatures used for species from cold waters and higher temperatures for species from warm waters. However, this is not always the case, as demonstrated by different temperature preferences for the same species in different studies.

Two studies involving Brazilian species were found: *H. malabaricus* (Ribas et al. 2016) and *A. lacustris* (Levy-Pereira et al. 2023). Both studies isolated cells from the head kidney, but the specific culture conditions differed. For *H. malabaricus*, the authors isolated macrophages, cultured them using L-15 with 2% FCS, and incubated them at a temperature range of 20 to 22°C. In contrast, for *A. lacustris*, the authors isolated leukocytes, cultured them using RPMI with 10% FCS, and incubated them at 25°C. The differences in culture conditions between the two species highlight the species-specific characteristics researchers must consider when working with fish leukocytes.

Hepatocytes

As liver cells, hepatocytes possess unique characteristics distinguishing them from other commonly used cells in *in vitro* experiments. Notably, hepatocytes exhibit a higher production of enzymes, which can influence various culture parameters such as autolysis risk and reduced need for antibiotics. Many protocols recommend perfusion with solutions containing enzymes and anticoagulants to decrease the presence of erythrocytes, thrombocytes, and the risk of autolysis. While CO_2 incubation was not commonly used in most experiments, adding HEPES buffer appeared necessary.

Some studies in the literature utilized Percoll or Ficoll solutions in hepatocyte isolation. Still, most directly seeded the cells after enzymatic digestion, incubated them overnight, and washed away non-adherent cells.

The most commonly used media for hepatocyte culture were L-15 and M199 (Table 4). However, other studies have described using RPMI, MHBSS, DMEM,

Table 4. Main conditions used for hepatocyte cultivation. [1]50% L-15, 35% DPBS, 15% Ham's F-12, final pH 7.2. [2]Virus production serum free-medium. [3]Salmon serum instead of FBS. [4]136.9 mmol L^{-1} NaCl, 5.4 mmol L^{-1} KCl, 0.8 mmol L^{-1} $MgSO_4$, 0.44 mmol L^{-1} $KH2PO_4$, 0.33 mmol L^{-1} $Na2HPO_4$, 5 mmol L^{-1} $NaHCO_3$, and 10 mmol L^{-1} HEPES. [5]High glucose, pH 7.4.

Species	Medium	FCS	Culture additives	Cell culture temperature	Reference
Sparus aurata	L-15	10%		18°C	Santacroce et al. (2011)
Oncorhynchus mykiss	L-15	10 %		18°C	Baron et al. (2012)
Danio rerio	LDF[(1)]	5% [(2)]	15 mM HEPES	NA	Maradonna et al. (2013)
Oncorhynchus mykiss	M199	10%		16°C	Connolly et al. (2015)
Salmo salar	L-15	10% [(3)]	1% glutamax and 1% penicillin-streptomycin-amphotericin (10,000 units mL^{-1} potassium penicillin 10,000 mcg mL^{-1} steptomycin sulfate and 25 µg mL^{-1} amphotericin B)	15 or 20°C	Olsvik et al. (2016)
Oncorhynchus mykiss	M199	10%		16°C	Bermejo-Nogales et al. (2017)
Hoplias malabaricus	RPMI 1640	5%	40 mg L^{-1} gentamicin sulfate	24°C	Silva et al. (2017)
Oncorhynchus mykiss	Modified HBSS[(4)]	-	1% defatted BSA, 3 mmol L^{-1} glucose, 2% MEM essential amino acid mixture, 1% MEM non-essential amino acid mixture, and 1% antibiotic antimycotic solution (1X)	18°C	Seite et al. (2019)
Ctenopharyngodon idellus *Oreochromis niloticus*	DMEM[(5)]	10%	with penicillin-streptomycin solution (100 U mL^{-1})	28°C	Jiao et al. (2020)
Ctenopharyngodon idellus	M199	10%		25°C	Cui et al. (2021)
Sparus aurata	L-15	10%	1% penicillin (10.000 mL^{-1}) / streptomycin (10 mg mL^{-1}) and amphotericin B (25 µg mL^{-1})	18°C	Bramatti et al. (2023)

or a custom medium called LDF (composed of 50% L-15, 35% DPBS, and 15% Ham's F-12, with a final pH of 7.2).

Regarding FCS concentrations, minimal variation was observed among the experiments, with 5 or 10% being the most commonly used concentrations. However, in the case of *Salmo salar*, Olsvik et al. (2016) used serum from the same species as a substitute for FCS.

One notable feature of hepatocytes is their lower requirement for antibiotics in culture. Half of the papers on fish hepatocyte culture reported no use of antibiotics or antifungal agents. In contrast, others described using a commonly employed commercial combination of penicillin, streptomycin, and amphotericin or, in some cases, gentamicin alone or as a substitute for streptomycin.

Compared to other cell lines in this review, the consulted literature demonstrated more consistent temperature variations for hepatocyte culture among different fish species. For the most commonly studied species, *O. mykiss*, the temperature ranged from 16 to 18°C, similar to *S. salar*, where incubation temperatures ranged from 15 to 20°C.

Specifically, in a study on hepatocytes from *H. malabaricus* conducted by Silva et al. (2017), RPMI was used as the medium, enriched with 5% FCS and 40 mg L^{-1} gentamicin sulfate, and the cells were incubated at 24°C. For another species of importance in Brazilian aquaculture, *O. niloticus*, hepatocytes were cultured by Bermejo-Nogales et al. (2017) using M199 with 10% FCS and no additional additives, incubated at 16°C. In the latter case, it is essential to note the relatively low temperature used for incubating the hepatocytes. It may introduce discrepancies for fish of the same species reared in tropical regions accustomed to warmer temperatures.

Myoblasts

As precursor cells that differentiate into myocytes, myoblasts play a significant role in studying muscle development and meat production. Enzymatic digestion of muscle tissue, with or without subsequent nylon mesh filtering, is a common method for obtaining myoblasts. During the cell isolation process, horse serum is frequently used, and for culture, many authors utilize poly-lysine-coated plates to enhance cell adhesion.

Table 5. Essential parameters found for myoblast cultivation. [1]Earle's Balanced Salt. [2]DMEM, 9 mmol L^{-1} $NaHCO_3$, 20 mmol L^{-1} HEPES, pH 7.4. [3]Vol/Vol 1:1. [4]9 mM $NaHCO_3$ (S5761), 20 mM HEPES (H3375), pH 7.4.

Species	Medium	FCS	Culture additives	Incubation temperature	Reference
Oncorhynchus mykiss	DMEM or F10	10%	9 mM $NaHCO_3$, 20 mM HEPES, and antibiotic-antimycotic cocktail	18°C	Seiliez et al. (2012)
Oncorhynchus mykiss	EBSS[(1)]	10%	MEM vitamins solution (Invitrogen, 11120-037), MEM essential amino acid mixture (Invitrogen, 11130-036), and MEM non-essential amino acid mixture100 U mL^{-1} penicillin, 100 µg mL^{-1} streptomycin, 0.25 g mL^{-1} fungizone at pH 7.4	18°C	Seiliez et al. (2012)
Devario cf. aequipinnatus	DMEM	10%	9 mM $NaHCO_3$, 20 mM HEPES, 1% v/v penicillin/streptomycin/fungizone	26°C	Froehlich et al. (2013)
Piaractus mesopotamicus	DMEM	10%	1% antibiotics	28°C	Duran et al. (2015)
Piaractus mesopotamicus	DMEM	10%		28°C	Duran et al. (2019)
Oncorhynchus mykiss	DMEM[(2)]	10%	1% antibiotic mixture	18°C	da Silva Duran et al. (2020)
Sebastes schlegelii	DMEM/F12:L-15[(3)]	20%	1% antibiotics, 1% NEAA (Non-essential amino acid), 10 ng mL^{-1} bFGF (basic fibroblast growth factor) and 10 µg mL^{-1} insulin	24°C	Kong et al. (2021)
Piaractus mesopotamicus	DMEM[(4)]	10%	1% antibiotics	28°C	Duran et al. (2022)

Based on the consulted literature, DMEM was myoblast culture's most commonly used medium (Table 5). However, some authors utilized EMEM, F10, F12, or L-15. Only one study employed 20% FCS among the papers, while the rest used 10% FCS.

Regarding media additives for myoblast culture, only one study (Duran et al. 2019) did not describe using any additives. In all other studies, additives were utilized, primarily antibiotics and antifungal agents, often referred to as an antibiotic cocktail or mixture. Additionally, several studies included 9 mM $NaHCO_3$ and HEPES, indicating a higher need for pH maintenance in myoblast cultures than in other cell types. Furthermore, amino acids were added to the media in two papers, and one study included basic fibroblast growth factor and insulin.

Regarding Brazilian studies, three papers focused on myoblasts from *Piaractus mesopotamicus*, an important species in Brazilian aquaculture (Duran et al. 2015, 2019, 2022). In all three studies, DMEM was used as the medium, with or without antibiotics. The incubation temperature was consistently set at 28°C. This higher temperature requirement indicates the need for warmer temperatures for Neotropical species.

CONCLUSION

In vitro cultivation techniques offer several applications and are vital in various scientific pursuits. It is crucial to recognize that each cell type possesses specific nutritional and thermal requirements that directly impact its optimal development, underscoring the significance of prior knowledge about the chosen cell model.

Despite the country's rich fish diversity, studies employing cell culture techniques in Brazil are relatively scarce, and this could be attributed to the limited dissemination of the advantages associated with *in vitro* approaches. However, cell culture holds significant promise for future investigations, offering ethical, practical, and cost-related advantages in analysis. Furthermore, aquaculture production can greatly benefit from research aimed at sector improvements, which can use *in vitro* cultivation as a refinement method before conducting *in vivo* assessments.

In conclusion, harnessing the potential of cell culture methodologies in Brazil can pave the way for innovative and impactful research, particularly in the context of environmental contaminants and the preservation of native species. Educating researchers about the manifold benefits of *in vitro* techniques can foster a deeper understanding and appreciation for these invaluable tools in advancing scientific knowledge and addressing critical environmental challenges.

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