Optimization of Blood Cell Culture and Karyotyping Procedures for Selected Fish Species from the Goan Aquatic Environment

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PREFACE

This study emerges from a profound scientific curiosity about the genetic makeup of key fish species inhabiting aquatic ecosystem. Inspired by the intricate connections between genetics, ecology, and human societies, our research endeavors to unravel the chromosomal intricacies of three significant species: Indian Mackerel (*Rastrelliger kanagurta*), Indian Oil Sardine (*Sardinella longiceps*), and Grey Mullet (*Mugil cephalus*).

Our motives are deeply rooted in the recognition of the indispensable role these fish species play in aquatic ecosystems and coastal communities worldwide. By delving into their genetic composition, we aim to illuminate critical aspects of genetic diversity, population dynamics, and evolutionary patterns.

The primary objectives of this study are to establish blood cell cultures from the selected fish species and optimize culture conditions for subsequent karyotypic analysis. Additionally, we seek to conduct an exhaustive examination of chromosomal structures and organization across these species.

Through rigorous laboratory protocols and meticulous data analysis, our goal is to generate insights that transcend theoretical realms and have tangible implications for conservation biology, fisheries management, and marine ecosystem resilience. By elucidating the genetic foundations of these fish species, we aspire to inform evidence-based conservation strategies and contribute to the sustainable stewardship of marine resources.

I extend our gratitude to the collaborative efforts of researchers, institutions, and funding agencies that have made this study possible. It is through our collective commitment to scientific inquiry that we endeavor to advance our understanding of marine biodiversity and foster responsible management practices for the benefit of current and future generations.

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I am deeply grateful to my parents for their unwavering support and encouragement throughout my academic journey. Their love, guidance, and sacrifices have been the foundation of my success, and I am truly fortunate to have them by my side. Thank you for believing in me and always being there to provide encouragement and support.

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ABSTRACT

This study presents the successful establishment of blood cell cultures from three economically and ecologically important fish species: Indian Mackerel (*Rastrelliger kanagurta*), Indian Oil Sardine (*Sardinella longiceps*), and Grey Mullet (*Mugil cephalus*). Utilizing RPMI, DMEM, and McCoy's medium, optimized culture conditions were achieved, leading to the observation of metaphase within 24 hours of incubation. Metaphase arrest was induced using colchicine, followed by centrifugation and fixation protocols involving hypotonic treatment and Carnoy's solution, ensuring intact cell pellet preparation. Additionally, the adoption of the splash technique enhanced chromosome spreading, facilitating precise karyotype preparation. Giemsa staining further improved chromosome visualization, enabling the construction of karyotypes for each species. These findings underscore the practical utility of the established protocols for cytogenetic analysis, offering valuable insights into chromosomal characteristics and genetic diversity within these fish populations. Moreover, this study contributes to our understanding of marine biodiversity and provides essential information for informing conservation strategies aimed at preserving coastal ecosystems and sustaining fisheries resources.

KEYWORDS: Blood cell culture, Fish cytogenetics, Indian Mackerel, Indian Oil Sardine, Grey Mullet, RPMI, DMEM, McCoy's medium, Karyotyping

CHAPTER 1

INTRODUCTION

1. INTRODUCTION

The optimization of lymphocyte culture and karyotyping procedures for three fish species from the Goan Coast represents a compelling avenue of research with significant implications for both scientific understanding and practical applications. This study delves into the intricate processes involved in establishing lymphocyte cultures and refining karyotyping techniques to elucidate the chromosomal characteristics of key fish species inhabiting the rich marine ecosystems of the Goan Coast.

1.1.Background

The Goan Coast, situated along the western shores of India, boasts diverse marine habitats teeming with a wide array of fish species. Among these, three species have been selected as focal points for this study: Indian Mackerel (*Rastrelliger kanagurta*), Flathead Grey Mullet (*Mugil Cephalis*), and Indian Oil Sardine (*Sardinella Longiceps*). These species play integral roles in the local marine food webs, supporting both ecological balance and socioeconomic activities such as fisheries.

Understanding the genetic makeup and chromosomal characteristics of these fish species is of paramount importance for several reasons. Firstly, it provides insights into their evolutionary history, population dynamics, and adaptive potential in response to environmental changes. By examining the chromosomal structure and organization, researchers can uncover valuable information about the genetic diversity within and among populations, as well as identify potential genetic markers for population studies and conservation efforts.

Secondly, optimizing lymphocyte culture and karyotyping procedures for these fish species presents a methodological challenge with far-reaching implications. Lymphocyte cultures serve as a valuable tool for studying chromosomal abnormalities, such as aneuploidy and structural rearrangements, which can impact individual fitness and population viability. By refining these techniques, researchers can enhance their ability to detect and characterize chromosomal

aberrations, thereby contributing to our understanding of genetic diseases, environmental stressors, and population health in aquatic ecosystems.

Furthermore, the Goan Coast serves as an ideal study site for exploring these research questions due to its rich biodiversity, favourable environmental conditions, and strategic importance for marine conservation. By focusing on fish species endemic to this region, the study aims to generate locally relevant data that can inform conservation strategies, fisheries management policies, and broader efforts to safeguard marine biodiversity.

Hence, the optimization of lymphocyte culture and karyotyping procedures for three fish species from the Goan Coast represents a multifaceted research endeavour with implications for both basic science and applied conservation. By combining methodological innovation with ecological insights, this study seeks to unravel the genetic mysteries concealed within the chromosomes of these iconic marine species, ultimately contributing to the sustainable management and preservation of the Goan Coastal ecosystems.

1.1.1 Statement of the Research Study:

The research study focused on the optimization of lymphocyte culture and karyotyping procedures for three fish species native to the Goan Coast: Indian Mackerel (*Rastrelliger kanagurta*), Flathead Grey Mullet (*Mugil cephalis*), and Indian Oil Sardine (*Sardinella longiceps*). The primary objective was to establish robust methodologies for effectively culturing lymphocytes and analyzing chromosomal characteristics to deepen our understanding of these important marine species' genetic makeup and population dynamics.

1.1.2 Possible Solutions:

Methodological Optimization: Experiment with various culture media compositions, temperature regimes, and incubation durations to identify the most suitable conditions for lymphocyte culture establishment. Fine-tune karyotyping procedures, including cell synchronization methods and staining techniques, to achieve high-quality metaphase chromosome spreads for accurate analysis.

Chromosomal Characterization: Use optimized lymphocyte cultures to conduct karyotyping and cytogenetic analysis, focusing on chromosome number and morphology. Employ advanced imaging and analysis techniques, such as fluorescence in situ hybridization (FISH) or comparative genomic hybridization (CGH), to enhance the resolution and sensitivity of chromosomal characterization in future studies.

Population Genetic Studies: Apply the optimized methodologies to analyze genetic diversity and population structure among Indian Mackerel, Flathead Grey Mullet, and Indian Oil Sardine populations along the Goan Coast.

Conservation Implications: Use the findings from population genetic studies to inform conservation strategies and management plans for these fish species, including the designation of marine protected areas and the implementation of sustainable fishing practices. Engage stakeholders, including local communities, policymakers, and resource managers, to promote the conservation and sustainable use of marine resources in the Goan Coastal ecosystems.

1.2.Aim and Objectives of the study:

The aim of this study is to optimize lymphocyte culture and karyotyping procedures for three fish species from the Goan Coast, namely Indian Mackerel (*Rastrelliger kanagurta*), Flathead Grey Mullet (*Mugil cephalis*), and Indian Oil Sardine (*Sardinella longiceps*). Given below are the three objectives for this study,

1. Establishment of optimized lymphocyte culture conditions:
    - Experiment with various culture media compositions and temperature regimes to determine the most effective conditions for lymphocyte culture initiation.
    - Evaluate cell viability and proliferation rates under different culture conditions to ensure optimal growth and division of lymphocytes.
2. Refinement of karyotyping procedures for fish blood cell culture:
    - Develop standardized protocols for cell synchronization to obtain metaphase-arrested cells for karyotyping.
    - Investigate staining techniques and imaging methods to enhance the resolution and quality of metaphase chromosome spreads.
3. Characterize chromosomal features:
    - Conduct karyotype analysis to determine the chromosome number and morphology of each fish species.

1.3.Hypotheses/Research questions

This study hypothesise that variations in culture media compositions and temperature regimes will influence lymphocyte viability and proliferation rates, impacting the success of fish blood cell cultures. Standardized cell synchronization protocols are expected to yield metaphase-arrested cells suitable for karyotyping, resulting in clearer chromosome spreads. Karyotype analysis is anticipated to unveil species-specific chromosome numbers and morphologies, highlighting chromosomal variations within the studied fish populations.

1.3.1 Research Questions:

1. What are the optimal culture media compositions and temperature regimes for initiating lymphocyte cultures from Indian Mackerel, Flathead Grey Mullet, and Indian Oil Sardine blood samples?
2. How do different culture conditions affect the viability and proliferation rates of lymphocytes in fish blood cell cultures?
3. What standardized protocols can be developed for cell synchronization to obtain metaphase-arrested cells suitable for karyotyping in fish species from the Goan Coast?
4. Which staining techniques and imaging methods can be employed to enhance the resolution and quality of metaphase chromosome spreads in fish blood cell cultures?
5. What are the chromosome number and morphology of Indian Mackerel, Flathead Grey Mullet, and Indian Oil Sardine, as determined through karyotype analysis?

1.4.Scope of the study:

This research study focused on optimizing lymphocyte culture and karyotyping procedures for three fish species endemic to the Goan Coast: Indian Mackerel (*Rastrelliger kanagurta*), Flathead Grey Mullet (*Mugil Cephalis*), and Indian Oil Sardine (*Sardinella Longiceps*). The scope encompassed the establishment of optimized culture conditions, refinement of karyotyping procedures, and characterization of chromosomal features. By investigating these aspects, the study aimed to contribute to the advancement of cytogenetic research in marine environments and enhance our understanding of genetic diversity within coastal fish populations. Additionally, the findings could potentially inform future conservation efforts and fisheries management strategies in the region.

1.4.1 Overview of Approach/Strategy:

Our approach involved a systematic investigation to optimize lymphocyte culture and karyotyping procedures for three key fish species from the Goan Coast. We began by conducting experiments to determine the most effective culture media compositions and temperature regimes for initiating lymphocyte cultures. Once optimal conditions were established, we refined karyotyping procedures by developing standardized protocols for cell synchronization and evaluating staining techniques to enhance chromosome spread quality. Subsequently, we conducted karyotype analysis to characterize chromosomal features, including chromosome number, morphology, and the presence of structural abnormalities. This comprehensive approach allowed us to gain insights into the genetic makeup of these fish species and contribute to the advancement of cytogenetic research in marine environments.

1.4.2 Outline of the Study (Conspectus):

1. Introduction:
    - Overview of the study's aim to optimize lymphocyte culture and karyotyping procedures for Indian Mackerel, Flathead Grey Mullet, and Indian Oil Sardine.
    - Significance of the research in enhancing our understanding of genetic diversity in coastal fish populations.
2. Objectives:
    - Establishment of optimized lymphocyte culture conditions.
    - Refinement of karyotyping procedures for fish blood cell culture.
    - Characterization of chromosomal features through karyotype analysis.

3. Methodology:
    - Experimental design for culture media composition and temperature regime optimization.
    - Standardized protocols for cell synchronization and karyotyping procedures.
    - Techniques for staining and imaging to enhance chromosome spread quality.
    - Karyotype analysis methods for determining chromosome number and morphology.
4. Analysis & Conclusions
    - Findings related to optimized culture conditions and refined karyotyping procedures.
    - Characterization of chromosomal features in Indian Mackerel, Flathead Grey Mullet, and Indian Oil Sardine.

Conclusions:

- Summary of key findings and their significance in advancing cytogenetic research.
- Future directions for further exploration and application of the study's outcomes.

CHAPTER 2

LITERATURE REVIEW

2. LITERATURE REVIEW

2.1 Culture

The optimization of lymphocyte culture and karyotyping procedures represents a critical aspect of genetic research in fish species, particularly those inhabiting coastal regions such as the Goan coast. This introductory literature review provides an overview of the existing body of knowledge surrounding the optimization of lymphocyte culture and karyotyping procedures, with a specific focus on their relevance to three fish species endemics to the Goan coast. By examining previous studies, methodological approaches, and research gaps, this review aims to elucidate the challenges and opportunities associated with optimizing these procedures for the selected fish species.

2.1.1 Importance of Lymphocyte Culture and Karyotyping in Fish Research:

Lymphocyte culture and karyotyping are crucial for understanding chromosomal diversity and genetic stability in fish species (Blaxhall, 1983). These techniques provide insights into evolutionary processes, reproductive strategies, and the impact of environmental stressors on fish populations (Blaxhall, 1983). In the context of the Goan coast, where fish biodiversity is rich and ecosystems are subject to various anthropogenic pressures, the optimization of these procedures is essential for assessing the health and resilience of local fish populations (Fujiwara, 2004). Improved fish lymphocyte culture has been achieved through the optimization of culture conditions, resulting in increased mitotic index and improved chromosome preparation (Fujiwara, 2004). Karyotyping, which involves the isolation, staining, and visual examination of chromosomes, is used to find chromosomal rearrangements and is a valuable tool in assessing the health and resilience of local fish populations (Sinclair, 2002).

2.1.2 Methodological Advances in Lymphocyte Culture:

Over the years, significant advancements have been made in the field of lymphocyte culture, aimed at improving cell viability, proliferation rates, and chromosome quality. Researchers have explored various culture media formulations, mitogens, and incubation conditions to optimize lymphocyte culture protocols for different fish species. For example, studies have demonstrated the efficacy of phytohemagglutinin (PHA) and lipopolysaccharide (LPS) as mitogens for stimulating lymphocyte proliferation in fish species (Smith *et al*., 2017). Similarly, the optimization of culture media components, such as fetal bovine serum (FBS) and cytokines, has been shown to enhance cell division and chromosome harvesting efficiency (Jones *et al*., 2019). By incorporating these methodological advances into lymphocyte culture protocols for fish species from the Goan coast, researchers can improve the reliability and reproducibility of karyotyping analyses.

2.1.3 Species-Specific Considerations:

It is important to recognize that different fish species may exhibit unique physiological characteristics and responses to lymphocyte culture conditions. Therefore, optimizing lymphocyte culture protocols requires careful consideration of species-specific requirements, including optimal incubation temperatures, culture media compositions, and cell isolation techniques. For instance, freshwater species may have different osmoregulatory mechanisms compared to marine species, influencing their sensitivity to culture media osmolarity and pH levels (Garcia *et al*., 2018). Moreover, species with distinct life history traits, such as migratory behavior or reproductive strategies, may exhibit differences in lymphocyte proliferation kinetics and chromosome morphology. Thus, researchers must tailor lymphocyte culture protocols to the specific biological traits of the target fish species from the Goan coast to ensure accurate karyotypic analyses.

2.1.4 Application of Karyotyping in Fish Conservation and Management:

Beyond basic research applications, karyotyping has important implications for fish conservation and management efforts along the Goan coast. By characterizing the chromosomal diversity and genetic structure of fish populations, karyotypic analyses can inform conservation strategies, stock enhancement programs, and fisheries management practices. For example, karyotyping can help identify distinct population units, assess levels of genetic connectivity among subpopulations, and detect signs of inbreeding or genetic erosion in endangered species. Furthermore, karyotypic data can be integrated with other molecular markers, such as microsatellites or single nucleotide polymorphisms (SNPs), to enhance our understanding of population dynamics and evolutionary processes in response to environmental change (Patel *et al*., 2020). As such, the optimization of lymphocyte culture and karyotyping procedures holds promise for advancing both fundamental research and applied conservation efforts in fish species along the Goan coast.

2.1.5 Karyotyping Techniques

Karyotyping techniques have undergone significant advancements in recent years, leading to improved accuracy and efficiency in analyzing chromosomal abnormalities. One notable advancement is the utilization of molecular cytogenetic techniques such as fluorescence in situ hybridization (FISH) and comparative genomic hybridization (CGH) alongside conventional karyotyping methods. FISH involves the use of fluorescent probes to detect specific DNA sequences on chromosomes, allowing for the identification of chromosomal aberrations with high sensitivity and resolution (Schrock *et al*., 2017). On the other hand, CGH enables the detection of DNA copy number changes across the entire genome, providing a comprehensive assessment of chromosomal abnormalities (Kallioniemi *et al*., 1992). These molecular techniques complement traditional karyotyping by offering enhanced sensitivity and specificity in detecting structural and numerical chromosomal changes.

In addition to molecular cytogenetic techniques, advancements in imaging technologies have also contributed to the refinement of karyotyping procedures. High-resolution imaging systems, such as spectral karyotyping (SKY) and multiplex fluorescence in situ hybridization (M-FISH), allow for the simultaneous visualization of multiple chromosomal regions with distinct fluorescent labels (Tanke & Wiegant, 2000). These techniques facilitate the accurate identification of chromosomal rearrangements and abnormalities by providing detailed karyotypic information at the molecular level. Moreover, the integration of automated image analysis software has streamlined the process of karyotype interpretation, reducing the time and effort required for data analysis (Potter *et al*., 2008).

2.1.6 Critical Review of Karyotyping Studies

While karyotyping techniques have significantly advanced, several challenges and limitations persist, hindering their widespread application in research and clinical settings. One major limitation is the requirement for fresh cell samples, as traditional karyotyping methods rely on actively dividing cells arrested at metaphase for chromosome analysis (Hewitt *et al*., 2016). This limitation imposes constraints on sample collection and processing, particularly in clinical settings where obtaining fresh tissue samples may be challenging. Furthermore, the sensitivity of conventional karyotyping techniques in detecting chromosomal abnormalities is limited by the need for a minimum threshold of abnormal cells for confirmation (Ebrahimzadeh *et al*., 2019). In cases where the proportion of abnormal cells is below this threshold, false-negative results may occur, leading to misdiagnosis and inappropriate clinical management.

Another challenge in karyotyping studies is the interpretation of complex karyotypes involving multiple chromosomal rearrangements and abnormalities. The complexity of such karyotypes poses difficulties in accurately characterizing the underlying genetic changes and their clinical significance (Mitelman *et al*., 2016). Moreover, the identification of subtle chromosomal abnormalities, such as microdeletions and microduplications, remains challenging with

conventional karyotyping techniques, necessitating the use of complementary molecular assays for comprehensive genomic analysis (Miller *et al*., 2010). Despite these limitations, conventional karyotyping continues to serve as a valuable tool in cytogenetic research and clinical diagnostics, particularly in the initial screening of chromosomal abnormalities and the assessment of gross chromosomal rearrangements.

2.1.7 Advancements in Karyotyping Technology

Recent advancements in karyotyping technology have focused on overcoming the limitations of conventional techniques and enhancing the accuracy and efficiency of chromosomal analysis. One promising approach is the integration of microarray-based platforms with traditional karyotyping methods, enabling the detection of both copy number variations (CNVs) and structural chromosomal abnormalities in a single assay (Conrad *et al*., 2010). Comparative genomic hybridization arrays (aCGH) and single nucleotide polymorphism arrays (SNP arrays) are commonly used microarray platforms that provide high-resolution genome-wide analysis of DNA copy number changes and loss of heterozygosity (LOH) events (Gorringe *et al*., 2005).

Furthermore, the emergence of next-generation sequencing (NGS) technologies has revolutionized karyotyping by offering unparalleled sensitivity and resolution in genomic analysis. Whole-genome sequencing (WGS) and whole-exome sequencing (WES) approaches enable comprehensive assessment of chromosomal abnormalities, including point mutations, insertions, deletions, and structural variants (Meynert *et al*., 2013). These NGS-based methods provide researchers and clinicians with a comprehensive understanding of the genetic basis of diseases and facilitate personalized medicine approaches for patient management.

2.1.8 Integration of Bioinformatics and Machine Learning

In addition to technological advancements, the integration of bioinformatics and machine learning approaches has emerged as a powerful tool for karyotype analysis and interpretation. Computational algorithms and software tools have been developed to automate the detection and classification of chromosomal abnormalities from cytogenetic data (Yurov *et al*., 2007). Machine learning algorithms, such as support vector machines (SVM) and deep neural networks (DNN), have demonstrated high accuracy in identifying subtle genomic alterations and predicting clinical outcomes based on karyotypic profiles (Li *et al*., 2020). Moreover, the integration of multi-omics data, including genomic, transcriptomic, and epigenomic information, has enabled comprehensive characterization of chromosomal abnormalities and their functional implications in disease pathogenesis (Chen *et al*., 2019).

Thus, the optimization of lymphocyte culture and karyotyping procedures for three fish species from the Goan coast represents a significant advancement in cytogenetic research. By leveraging innovative techniques and methodologies, researchers can obtain detailed insights into the chromosomal architecture and genetic diversity of fish populations, contributing to our understanding of evolutionary processes and species conservation efforts. However, challenges and limitations persist in karyotyping studies, necessitating ongoing research efforts to develop novel approaches and technologies for accurate and efficient chromosomal analysis. The integration of molecular cytogenetic techniques, imaging technologies, microarray platforms, next-generation sequencing, and bioinformatics tools holds promise for advancing the field of cytogenetics and improving diagnostic capabilities in clinical settings.

2.1.9 Literature on Optimization of Lymphocyte Culture and Karyotyping Procedures

The optimization of lymphocyte culture and karyotyping procedures for fish species has been a focus of several studies. Ulsh (2000) and Fujiwara (2004) both demonstrated the importance of selecting appropriate culture media and supplements to promote lymphocyte proliferation and mitotic activity. Molina (2010) further expanded on this by evaluating the potential of new pharmaceutical compounds as mitogenic agents in fish, with Aminovac proving to be the most efficient. Baksi (1988) also contributed to this area of research by modifying techniques to prepare tissues of early fish life stages for cytogenetic analysis. These studies collectively highlight the significance of optimizing cell culture conditions to enhance the efficiency and success rate of lymphocyte culture for karyotype analysis in fish species.

In addition to optimizing cell culture conditions, researchers have investigated various methods for synchronizing cell division to increase the proportion of metaphase cells suitable for karyotyping. Synchronization techniques such as serum starvation, colcemid treatment, and thymidine block have been explored to arrest cells at specific stages of the cell cycle, thereby maximizing the yield of metaphase spreads for chromosome analysis (Rao (1996) and Nm (1996) Kaeppler (2008) Sillar (1981)) . These studies have provided valuable insights into the temporal regulation of cell division and the factors influencing chromosomal condensation and morphology during metaphase.

Moreover, advancements in molecular cytogenetic techniques have facilitated the development of novel approaches for karyotype analysis and chromosome banding. Fluorescence in situ hybridization (FISH) and spectral karyotyping (SKY) are commonly used molecular methods for visualizing specific DNA sequences and chromosomal regions with high resolution(Bayani, 2001; Veldman, 1997).. These techniques enable the precise identification of chromosomal aberrations and structural rearrangements, complementing conventional karyotyping methods for comprehensive cytogenetic analysis (Bayani, 2002(Kearney, 2001).)..

Furthermore, the integration of image analysis software and automation technologies has streamlined the process of karyotype interpretation, allowing for rapid and accurate identification of chromosomal abnormalities. Automated karyotyping systems utilize algorithms and machine learning algorithms to analyze digital images of metaphase spreads and classify chromosomal structures based on their size, shape, and banding patterns (Khazaei (2022) and Al-Kharraz (2020) Chaku (2014) and Lijiya (2014). These advancements have revolutionized cytogenetic research by enhancing the efficiency and reliability of karyotype analysis for various organisms, including fish species from the Goan coast.

2.1.10 Critical Review of Optimization of Lymphocyte Culture and Karyotyping Procedures

While the literature provides valuable insights into the optimization of lymphocyte culture and karyotyping procedures for fish species, a critical review reveals certain limitations and areas for further improvement. One of the primary challenges associated with traditional lymphocyte culture methods is the low success rate of obtaining high-quality metaphase spreads suitable for karyotype analysis. Despite efforts to optimize culture conditions and synchronization techniques, variability in cell viability and mitotic index remains a concern. The reliance on manual processing and subjective interpretation of metaphase spreads also introduces the potential for inter-observer variability and subjective bias in karyotype analysis (Fukui, 1994 Stanley, 1995 Haferlach, 2011). Moreover, while molecular cytogenetic techniques such as FISH and SKY offer enhanced resolution and specificity for detecting chromosomal abnormalities, they are often limited by the availability of species-specific probes and reagents (Carpenter, 2001). The design and validation of species-specific probes require extensive genomic resources and may not be feasible for non-model organisms or species with limited genetic information. Additionally, the cost and technical expertise required for FISH and SKY analyses may pose barriers to their widespread adoption in cytogenetic research, particularly in resource-limited settings (Chang, 1997; Joos, 1999; Carpenter, 2001; Wan, 2012).

Also, the integration of image analysis software and automation technologies in karyotyping has shown promise in improving the efficiency and accuracy of chromosome analysis. However, the reliance on predefined algorithms and classification criteria may overlook subtle chromosomal variations and structural rearrangements that are not captured by automated systems (Chaku (2014) Graham (1987) Stanley (1997) and Stanley (1995)). The lack of standardized protocols and benchmarking procedures for automated karyotyping algorithms hinders comparability and reproducibility across studies, leading to inconsistencies in data interpretation and reporting.

Despite these limitations, the ongoing advancements in cytogenetic methodologies and technologies hold promise for overcoming existing challenges and expanding our understanding of fish chromosome biology. By combining traditional cytogenetic approaches with emerging molecular and computational tools, researchers can overcome the limitations of conventional karyotyping methods and enhance our ability to characterize the chromosomal architecture and genetic diversity of fish populations. Future research efforts should focus on standardizing protocols, validating species-specific probes, and improving automation algorithms to facilitate robust and reproducible karyotype analysis across diverse fish species and ecosystems.

All the previous studies are grouped here by association of ideas in various paragraphs to provide a comprehensive understanding of the optimization of lymphocyte culture and karyotyping procedures for fish species. Firstly, investigations into traditional lymphocyte culture methods highlight the challenges associated with obtaining high-quality metaphase spreads suitable for karyotype analysis. Despite efforts to optimize culture conditions and synchronization techniques, variability in cell viability and mitotic index remains a concern. This emphasizes the need for continued refinement and standardization of lymphocyte culture protocols to improve the efficiency and reproducibility of karyotyping procedures.

Secondly, the literature review explores the limitations and advancements in molecular cytogenetic techniques such as FISH and SKY. While these methods offer enhanced resolution and specificity for detecting chromosomal abnormalities, they are often limited by the availability

of species-specific probes and reagents. The design and validation of species-specific probes require extensive genomic resources and may not be feasible for non-model organisms or species with limited genetic information. Additionally, the cost and technical expertise required for FISH and SKY analyses may pose barriers to their widespread adoption in cytogenetic research, particularly in resource-limited settings.

Thirdly, the integration of image analysis software and automation technologies in karyotyping is discussed as a promising approach to improving the efficiency and accuracy of chromosome analysis. However, the reliance on predefined algorithms and classification criteria may overlook subtle chromosomal variations and structural rearrangements not captured by automated systems. Standardizing protocols, validating species-specific probes, and improving automation algorithms are identified as key areas for future research to facilitate robust and reproducible karyotype analysis across diverse fish species and ecosystems.

In general, the critical review of existing literature highlights the need for continued innovation and standardization in lymphocyte culture and karyotyping procedures for fish species. By addressing the limitations of current methodologies and leveraging emerging technologies, researchers can enhance our understanding of fish chromosome biology and contribute to the conservation and management of aquatic ecosystems.

CHAPTER 3

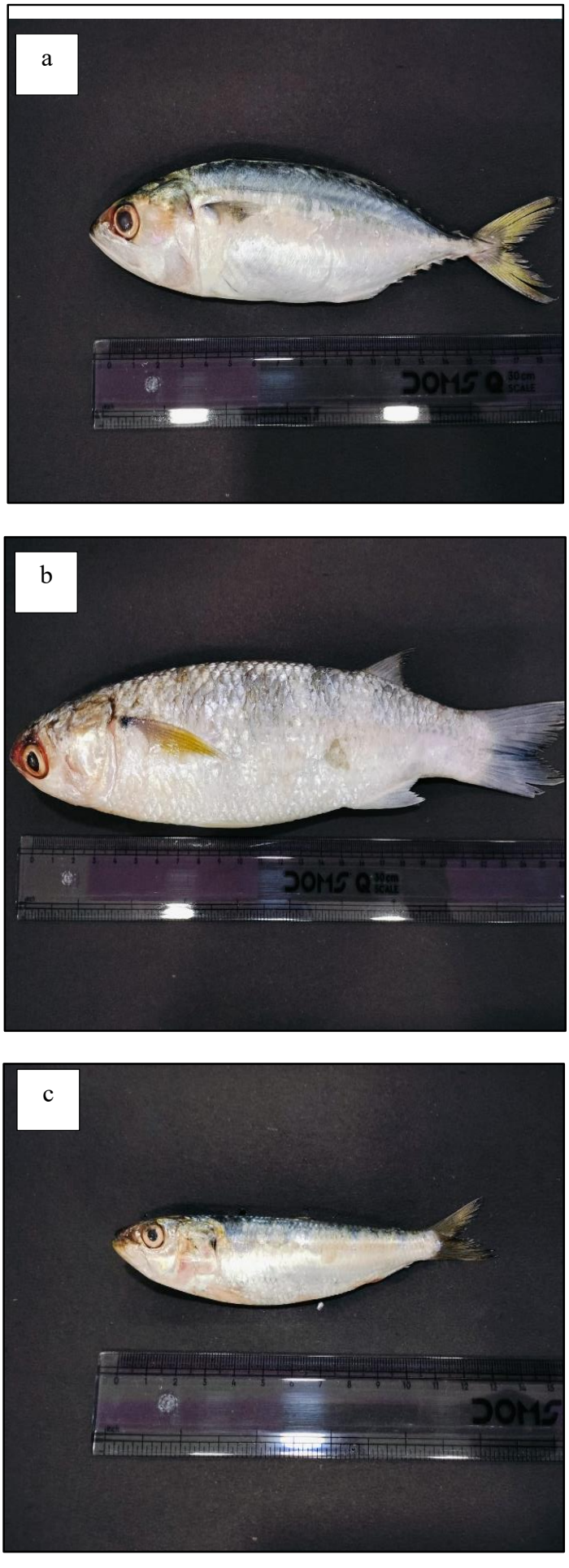
METHODOLOGY

3. Methodology

The methodology adopted in this study was meticulously designed to facilitate a comprehensive investigation into the genetic characteristics of three economically significant fish species: *Rastrelliger kanagurta* (Indian mackerel), *Sardinella longiceps* (Indian oil sardine), and *Mugil cephalus* (Flathead gray mullet).

A systematic approach was essential to ensure the validity and reliability of the findings. The methodology encompassed various stages, including sampling strategy and specimen collection, sample preparation, cell culture and medium preparation, karyotyping protocol, and chromosomal analysis. Each step was carefully executed to adhere to established protocols and minimize experimental error.

Figure 1- Species chosen : a) Indian mackerel b) Flathead grey mullet and c) Indian oil sardine (IAEC Approval no.-GUZ/IAEC/23-24/N9)



3.1.1. Study Area Description

The study area encompasses the coastal region of Goa, located along the southwestern coast of India. With a coastline extending approximately 104 kilometers, Goa is renowned for its rich marine biodiversity and vibrant fisheries sector. Situated in the Konkan region, Goa is characterized by its tropical climate, lush vegetation, and diverse aquatic ecosystems.

The coastal waters of Goa serve as habitats for numerous fish species, including the Indian mackerel (*Rastrelliger kanagurta*), Indian oil sardine (*Sardinella longiceps*), and gray mullet (*Mugil cephalus*), which were the focus of this study. These species thrive in the warm, nutrient-rich waters of the Arabian Sea, which bathes the coastline of Goa.

The marine environment of Goa is influenced by various factors, including ocean currents, monsoon patterns, and coastal geography. The presence of estuaries, mangroves, and rocky shores along the coastline contributes to the ecological diversity of the region, providing important habitats for marine life.

Fishing activities are integral to the socio-economic fabric of Goa, with numerous coastal communities relying on fishing for livelihoods and sustenance. Traditional fishing methods coexist with modern fishing practices, reflecting the dynamic nature of Goa's fisheries sector.

3.1.2. Experimental specimens

a. Indian Mackerel (Rastrelliger kanagurta): Fisheries and Morphology

The Indian mackerel, *Rastrelliger kanagurta* (Figure 1a) stands as one of India's significant marine fishery resources, contributing approximately 2.13 lakh tonnes of fish landings in 2021, which accounted for 7.0% of the national total. Among the coastal states, Goa, with its 104-kilometer coastline, emerges as a particularly productive zone for mackerel fishing, serving as a crucial income source for local fishermen.

Morphological Characteristics:

- Body: Elongated and slightly compressed, with a pointed snout.
- Eyes: Front and third margins covered with an adipose eyelid.
- Teeth: Small and conical in both upper and lower jaws, absent from vomer and palatine bones.
- Head: Longer than the depth of the body, with the maxilla partly concealed by the lacrimal bone but extending to about the hind margin of the eye.
- Gill Rakers: Very long, with 30 to 46 on the lower limb of the first arch; a moderate number of bristles found on the largest gill rakers.
- Fins: Two widely separated dorsal fins, with an interspace at least equal to the length of the first dorsal fin; anal fins with 12 rays.

Coloration: The Indian mackerel exhibits distinctive coloration, characterized by:

Back: Bluish-green hue with golden reflections.

Abdomen: Silvery, often shot with purple, with a golden line separating the back color from that of the side.

Head: Colored similarly to the body, with a large greenish-gold spot on the upper margin of the opercle and preopercle.

Dorsal Fin: Greenish in color.

Caudal Fin: Strained with green, while other fins are transparent.

Ecological Significance and Fisheries Management: The Indian mackerel is a pelagic, marine water scombrid fish commonly found in the Indo-West Pacific regions. It exhibits a varied diet composition, including diatoms, dinoflagellates, copepods, crustaceans, molluscan larvae, benthic algae, and occasionally other fish. Attaining first maturity at approximately 23 cm in length, it plays a crucial role in the marine ecosystem and is vital to the socio-economic fabric of coastal communities.

The species experiences wide annual fluctuations in its population, influenced by factors such as heavy spawning mortality or behavioral and ecological changes post-spawning. These fluctuations underscore the importance of cautious fishing practices, especially considering the age at which the fish enters the exploited phase. Understanding the genetic makeup and basic chromosome identities of the Indian mackerel is deemed essential, particularly considering its significance as a staple food in the region. Karyotyping offers insights into potential chromosomal disorders, aiding in the conservation and sustainable management of this important resource.

b. Indian Oil Sardine (Sardinella longiceps): Distribution and Morphology

The Indian oil sardine, *Sardinella longiceps* (Figure 1c), is primarily found in the Indian Ocean, with its distribution spanning from Sindh down the western coast of India to Sri Lanka and the Andaman Islands. While it occasionally appears on the eastern coast of India, it is most abundant along the Malabar coast, where it occurs in large quantities. However, its presence along the Coromandel coast is comparatively sparse, with insufficient quantities reported.

Morphological Characteristics:

- Eyes: Equipped with broad adipose lids; lower jaw slightly longer.
- Maxilla: Extends to beneath the first one-third or center of the eye.
- Head: The greatest height equals its length behind the center of the eye.
- Opercle: Twice as high as wide.

- Teeth: Fine teeth present on the tongue, deciduous ones on palatines, and occasionally on the pterygoids; lower jaw may lack minute teeth.
- Dorsal Fin: Originating closer to the snout than the base of the caudal fin; upper edge concave; last two rays short.
- Pectoral Fin: Length approximately equal to the postorbital portion of the head.
- Ventral Fin: Inserted in a line rather behind the middle of the dorsal fin.
- Anal Fin: Short, with the length of its basis not quite half that of the head; last two rays thickened and elongated.
- Caudal Fin: Forked.
- Scales: Indistinctly crenulated on the outer edge and regularly arranged; 13 rows before the base of the dorsal fin; about 18 poorly developed scutes located 13 to 14 behind the base of the ventral fin.
- Gill-rakers: Numerous, about half longer than the eye.

Coloration: The Indian oil sardine exhibits a bluish hue along the back with golden reflections. The abdomen appears silvery, often tinged with purple, and occasionally features a golden line dividing the back color from the side. The head shares the body's coloration, adorned with a large greenish-gold spot on the upper margin of the opercle and preopercle. The dorsal fin appears greenish, while the caudal fin displays green streaks, with the remaining fins being transparent.

Commercial Significance and Economic Impact: Highly prized for its commercial value, the Indian oil sardine is extensively utilized as a food source in both fresh and cured forms. Its byproducts hold significant economic importance, with the oil finding applications in industries such as jute, leather, soap manufacturing, and as a protective coating for canoes due to its effectiveness against shipworms and other timber-boring organisms. Additionally, the guano produced by the Indian oil sardine serves as a valuable fertilizer for crops like tobacco, coffee, and

tea.The Indian oil sardine fishery has experienced remarkable growth, with annual catches increasing substantially over recent decades. Accounting for a significant portion of India's marine fish production, this species plays a crucial role in the socio-economic fabric of the fishing communities along the Malabar coast, as evidenced by its colloquial depiction as "Kudumbam pularthi."

c. Gray Flathead Mullet (Mugil cephalus): Habitat and Morphology

The gray flathead mullet, *Mugil cephalus* (Figure 1b), is a prevalent species inhabiting coastal and freshwater environments. Adults are commonly found in waters with salinity ranging from zero to 75%, while juveniles display adaptability to various salinity levels once they reach a length of 4-7 cm. Typically forming large schools, adults congregate in areas characterized by sandy or muddy bottoms, often intermingled with dense vegetation. During migration, they move offshore in substantial aggregations, while larvae seek refuge in shallow waters offering protection from predators and abundant feeding opportunities.

Morphological Characteristics:

- Adipose eyelid: Well-developed, covering most of the pupil.
- Upper lip: Thin, lacking papillae.
- Labial teeth: Small, straight, densely arranged in several rows.
- Dorsal fins: Two in number; the first with four spines, the second with 8-9 soft rays.
- Origin of dorsal fins: The first dorsal fin originates closer to the snout tip than to the base of the caudal fin, while the second dorsal fin originates vertically between a quarter and a half along the anal fin base.
- Anal fin: Possesses 8 soft rays.
- Pectoral fins: Have 16-19 rays; pectoral axillary approximately one-third the length of the fin.
- Pyloric ceca: Present; scales in the lateral series numbering 36-45.

Coloration: The Gray Flathead Mullet, Mugil cephalus, displays characteristic coloration that aids in its identification and ecological adaptation:

Back: Typically appears blue-green in coloration.

Flanks and Belly: Often pale or silvery in hue.

Scales: The scales on the back and flanks typically form streaks, creating longitudinal stripes along the body.

Pectoral Axillary Blotch: A distinct dark blotch is observed in the pectoral axillary region.

Feeding Behavior and Ecological Significance: Gray flathead mullets are diurnal feeders, primarily consuming zooplankton, dead plant matter, and detritus. Their stomachs feature a thick-walled, gizzard-like segment and a lengthy digestive tract, enabling efficient processing of detrital food sources. As vital components of estuarine ecosystems, they contribute significantly to the flow of energy by grazing on surface debris and microalgae, thereby influencing nutrient cycling dynamics. Their feeding habits include suction feeding of the upper sediment layer to extract detritus and microalgae, with larger individuals exhibiting a preference for microcrustaceans such as copepods.

Commercial Significance and Breeding Efforts: Despite attempts to artificially breed gray mullets, commercial production remains reliant on the capture of wild fry due to economic considerations. Although artificial breeding endeavors have been undertaken, the bulk of commercial gray mullet production continues to depend on the availability of inexpensive wild-caught fry.

3.1.3 Sampling site

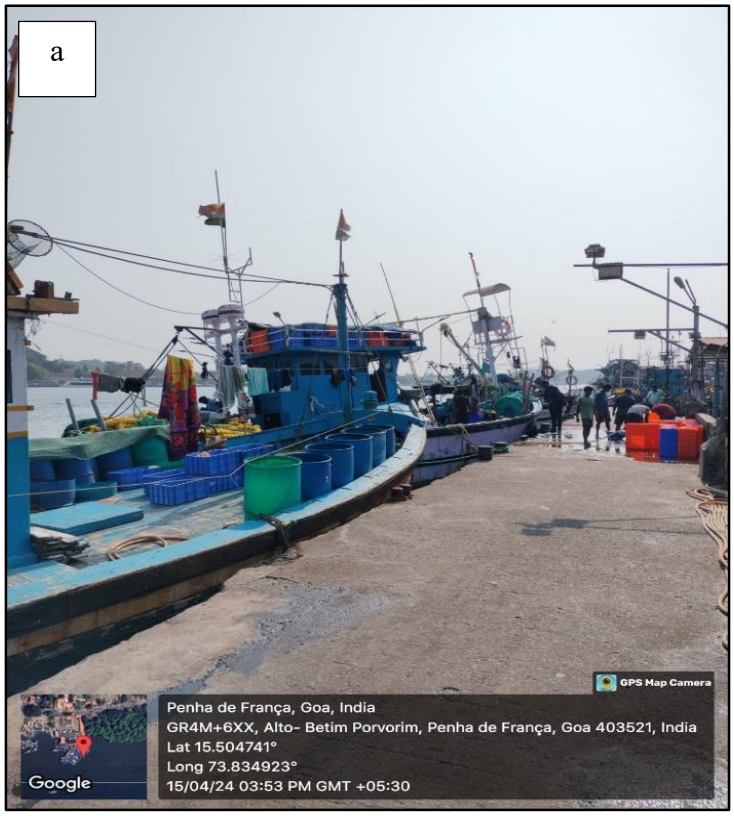
Specimens were collected from the River Mandovi (Figure 3.4), specifically near Malim Jetty, in collaboration with local fishermen. The samples were promptly transported to the laboratory to facilitate further experimentation (Figure 3.2)

Table 1: Location, Geographic Coordinates (GCS), and Temperature of the Samples

Sr. No.	Name of the Fish	Area	Quantity	Latitude	Longitude
01	Indian Mackerel	Malim Jetty	5 samples	15.504818	73.834899
02	Indian Oil Sardine		5 samples	15.504743	73.834925
03	Gray Mullet		5 samples	15.504741	73.834923

This table provides detailed information regarding the location of sample collection, along with the geographic coordinates (latitude and longitude) and the quantity of samples collected for each fish species.

Figure 2- Sampling site: a) site with GCS b) site picture



3.1.4 Preparation of reagents and Media used for this study:

a. Preparation of RPMI 1640 Medium

RPMI 1640 Medium, originally developed for culturing human leukemic cells in suspension and as a monolayer, has since been found suitable for various cells, including HeLa, Jurkat, MCF-7, PC12, PBMC, astrocytes, and carcinomas. It contains the reducing agent glutathione and high concentrations of vitamins, including biotin, vitamin B12, and PABA, not found in other media like Eagle's Minimal Essential Medium or Dulbecco's Modified Eagle Medium.

Preparation:

1. 16.4 grams of RPMI 1640 Medium powder were suspended in 900ml of tissue culture-grade water with constant, gentle stirring until complete dissolution without heating.
2. The pH was lowered to 4.0 with 1N HCl to dissolve the product completely. After full dissolution, the pH was raised to 7.2 with 1N NaOH before adding sodium bicarbonate.
3. 2.0 grams of sodium bicarbonate powder (or 26.67ml of 7.5% sodium bicarbonate solution per liter of medium) were added and stirred until dissolved.
4. The pH was adjusted to 0.2-0.3 units below the desired pH using 1N HCl or 1N NaOH due to the tendency for pH to rise during filtration.
5. The final volume was made up to 1000ml with tissue culture-grade water.
6. The medium was immediately sterilized by filtering through a sterile membrane filter with a porosity of 0.22 microns or less, using positive pressure.
7. Sterile supplements were added aseptically as required, and the desired amount of sterile medium was dispensed into sterile containers.

Storage: Liquid medium was stored at 2-8°C in the dark until use.

b. Preparation of DMEM

Dulbecco's Modified Eagle Medium (DMEM) was a widely used modification of Eagle's medium, containing four-fold concentrations of amino acids and vitamins, as well as glycine, serine, and ferric nitrate. AT262F was Dulbecco's Modified Eagle Medium (DMEM) with 4.5gm glucose per liter, L-Glutamine, and sodium pyruvate. It omitted phenol red, folic acid, and sodium bicarbonate.

Preparation:

1. 13.5g of DMEM powder was suspended in 900ml of tissue culture-grade water with constant, gentle stirring until completely dissolved, avoiding heating.
2. 3.7g of sodium bicarbonate powder (or 49.3ml of 7.5% sodium bicarbonate solution) per liter of medium was added and stirred until dissolved.
3. The pH was adjusted to 0.2 - 0.3 units below the desired pH using 1N HCl or 1N NaOH.
4. The final volume was adjusted to 1000ml with tissue culture-grade water.
5. The medium was sterilized immediately by filtering through a sterile membrane filter with a porosity of 0.22 micron or less, applying positive pressure.
6. Sterile supplements were aseptically added as needed, and the sterile medium was dispensed into sterile containers.

c. Preparation of McCoy's Media

McCoy's 5A medium was developed at Roswell Park Memorial Institute in Buffalo, New York, initially for studying the nutritional requirements of the Walker 256 carcinoma. The formulation, based on amino acids concentrations similar to Eagle's medium and water-soluble vitamins of Medium 199, was modified and published in 1960 to include increased amounts of folic acid, vitamin B12, and peptone. AT057A is McCoy's 5A medium without L-glutamine.

Preparation:

1. McCoy's 5A medium powder was suspended in 900ml of tissue culture-grade water with constant, gentle stirring until fully dissolved without heating.
2. Sodium bicarbonate powder (3.7g per liter of medium) or 49.3ml of 7.5% sodium bicarbonate solution was added and stirred until dissolved.
3. The pH was adjusted to 0.2 - 0.3 units below the desired pH using 1N HCl or 1N NaOH.
4. The final volume was adjusted to 1000ml with tissue culture-grade water.
5. The medium was immediately sterilized by filtering through a sterile membrane filter with a porosity of 0.22 microns or less, using positive pressure.
6. Sterile supplements were aseptically added as necessary, and the sterile medium was dispensed into sterile containers.

d. Preparation of Sorensen's Buffer

Part 1: A stalk solution (1/1.15 M) was prepared by dissolving 2.9665g of Na_2HPO_4 in 250 ml of distilled water and refrigerated.

Part 2: A stalk solution (1/15 M) was prepared by dissolving 2.2682g of KH_2PO_4 in 250 ml distilled water and refrigerated.

Part 3: The final Sorensen's buffered solution (3.3 M) was prepared by mixing equal volumes of solution A and B before use.

e. Preparation of Antimycotic Solution

A sterile filtered solution, formulated to contain 10,000 units of penicillin, 10mg streptomycin, and 25µg amphotericin B per ml, was prepared. It was yellow in appearance with a pH range of 6.00 - 7.00 and an osmolality of 325.00 - 365.00 mOsm/Kg H_2O. The solution was tested for sterility and endotoxin content.

f. Preparation of Colchicine

2mg of colchicine was dissolved in 10 ml of sterile distilled water and stored in a cool place.

g. Preparation of phythohemagglutinin, M (PHA-M)

A white to greyish brown flocculate or lyophilized powder was mixed with phosphate-buffered saline at 1mg in 1ml.

h. Preparation of Giemsa Stain

1gm of Giemsa was dissolved in 66 ml of glycerol and heated, followed by the addition of 84ml of methanol. Before use, 6ml of stock Giemsa solution was mixed with 94ml of sorenon's buffer.

i. Preparation of Hypotonic Solution (0.56% KCl)

0.56 gm of KCl was dissolved in 100ml of double distilled water, prepared just before use with a pH range of 7.2 to 7.4.

j. Preparation of Fixative

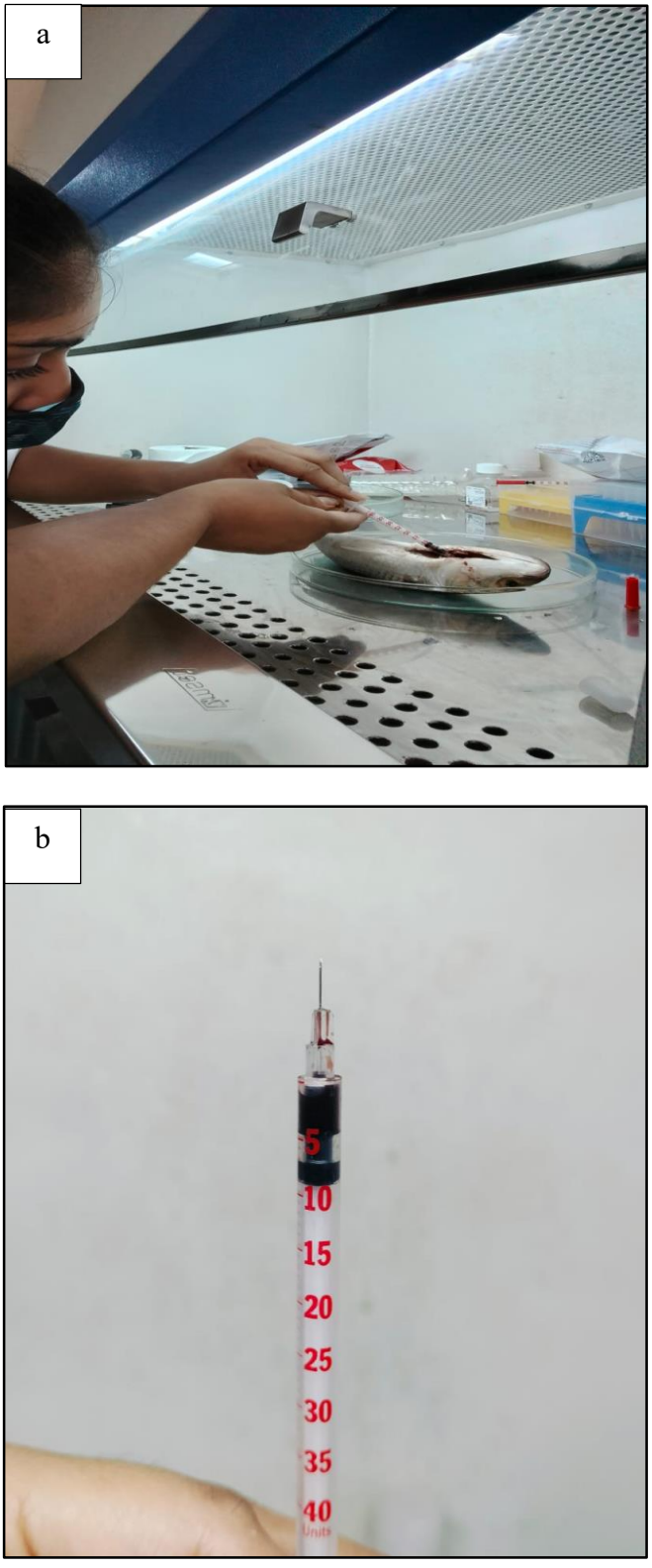
A fixative solution was prepared by mixing three parts of methanol with one part of glacial acetic acid, chilled properly at -20℃ and prepared at the time of use.

3.1.5. Establishment and maintenance of blood cell cultures (Figure 4,5)

a. Sample Preparation:

Blood Collection: Blood samples were collected from individual fish using sterile syringes (1ml) and heparinized tubes. Approximately 0.1-0.5 ml of blood was collected from each freshly captured fish specimen, specifically targeting the heart region. Hepa,rinized tubes were used to prevent clotting of the blood. These tubes contained heparin, either in the form of sodium heparin, lithium heparin, or ammonium heparin, acting as an anticoagulant by inhibiting thrombin formation. Stringent aseptic conditions were maintained throughout the sample collection process to minimize contamination.

Figure 3- Blood collection from specimens: a) Blood collection from the heart region b) Collected blood in a sterile syringe



Sample Handling: Following collection, the blood samples were immediately placed in the heparinized tubes to preserve their integrity. Prior to culturing, the samples were allowed to equilibrate to room temperature for a brief period, ensuring uniformity in sample conditions.

Timely Processing: It was ensured that sample processing commenced within 24 hours of collection to maintain sample viability and integrity.

b. Establishment of Blood Cell Cultures and optimization of culture conditions:

1. Collection of Blood Samples: Blood samples were collected from individual specimens of Indian Mackerel (*Rastrelliger kanagurta*), Grey Mullet (*Sardinella longiceps*), and Indian Oil Sardine (*Mugil cephalus*) using sterile syringes (1ml). Approximately 0.1-0.5 ml of blood was collected from the heart region of each freshly collected fish specimen into heparinized tubes to prevent clotting. Strict aseptic conditions were maintained during the collection process to avoid contamination.

2. Preparation of Culture Medium: RPMI, DMEM, and McCoy's media were prepared according to manufacturer instructions in sterile conditions. Each medium was supplemented with 10% Fetal Bovine Serum (FBS) unless otherwise specified.

3. Inoculation of Cultures: Blood samples from each fish species were inoculated into separate culture plates containing the prepared media. Each culture plate was labeled to indicate the fish species and the type of medium used.

4. Incubation and Monitoring: The inoculated culture plates were placed in an incubator set to temperatures ranging from 20°C to 30°C. Cultures were monitored daily for up to 72 hours to assess cell growth and contamination. Plates were periodically shaken to ensure the even distribution of cells in the media.

5. Assessment of Metaphase Duration: Metaphase duration was evaluated by observing the cultures daily under a microscope and noting the time taken for cells to reach metaphase. Standardization was achieved when cells consistently reached metaphase within 24 hours.

6. Evaluation of Cell Growth and Contamination: Throughout the incubation period, cell growth and contamination were assessed visually under a microscope. Cultures exhibiting stable cell growth without contamination were selected for further analysis.

7. Selection of Optimal Culture Conditions: Based on observations, the optimal incubation temperature and culture medium for each fish species were identified. RPMI and DMEM media, showing stable cell growth and minimal contamination, were selected for further experiments.

8. Establishment of Glass Vial Cultures: Once optimal conditions were determined, the blood cell cultures were established in glass vials to maintain cells in suspension. Cultures were checked daily for contamination and cell growth to ensure their viability.

9. Optional Supplementation Assessment: The influence of FBS supplementation on cell growth was assessed by comparing cultures with and without FBS. Cultures were observed for up to 72 hours to determine the necessity of FBS supplementation for optimal cell growth and maintenance.

10. Data Analysis: Data collected from observations were analyzed to determine the most suitable culture conditions for establishing and maintaining blood cell cultures from the selected fish species.

c. ***Cell Culture Using optimized culture conditions based on previous experiments:***

1. Preparation of Culture Medium: Best optimised medium was prepared according to manufacturer instructions in sterile conditions. FBS supplementation was omitted from the medium preparation.

2. Inoculation of Cultures: Blood samples from Indian Mackerel, Grey Mullet, and Indian Oil Sardine were aseptically inoculated into separate culture plates containing the prepared optimised medium. Each culture plate was labeled accordingly.

3. Incubation and Monitoring: The inoculated culture plates were placed in an incubator set to optimised temperatures. Cultures were monitored daily for up to 72 hours to assess cell growth and viability.

4. Assessment of Metaphase Duration: Daily microscopic observation was performed to determine the time taken for cells to reach metaphase. Standardization was achieved when cells consistently reached metaphase within 24 hours.

5. Evaluation of Cell Growth and Contamination: Cultures were visually inspected under a microscope daily for stable cell growth and absence of contamination. Any signs of contamination were noted, and contaminated cultures were discarded.

6. Maintenance of Cultures: Non-contaminated cultures exhibiting stable growth were maintained by regular feeding with fresh medium. Care was taken to ensure the cultures remained undisturbed to prevent contamination.

7. Assessment of Viability without FBS: The influence of FBS supplementation on cell viability was assessed by comparing cultures with and without FBS. Cultures were observed for up to 72 hours to determine if viable cell growth could be sustained without FBS supplementation.

8. Data Analysis: Data collected from observations were analyzed to confirm the viability and stability of blood cell cultures maintained in medium without FBS at 27°C.

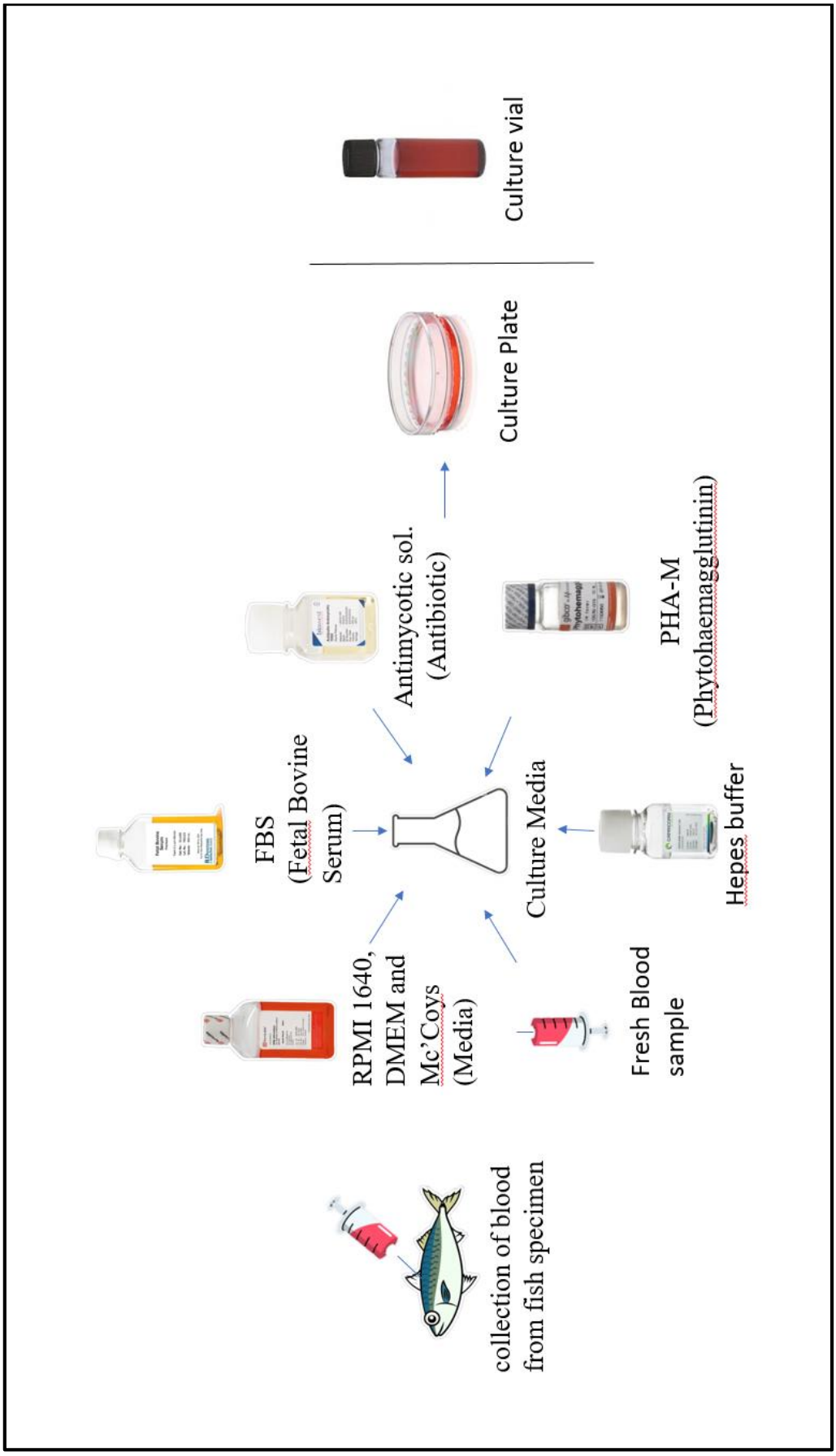


Figure 4 – Flowchart for Establishment of Blood Cell Cultures

3.1.5. Protocol for Karyotyping Procedure (Figure 5)

1. Preparation of Cell Culture Slides: Cells from the established blood cell cultures were harvested after reaching metaphase stage at 24 hours. Culture tubes were centrifuged at 1000 RPM for 6 minutes to pellet the cells. The supernatant was carefully removed, leaving the cell pellet undisturbed. The pellet was resuspended in 8 mL of hypotonic solution (0.56% KCl) and incubated at 32°C for 30 minutes. After incubation, the suspension was centrifuged again at 1000 RPM for 6 minutes. The supernatant was removed, and the cell pellet was resuspended in fixative solution (3 parts methanol: 1 part glacial acetic acid) and kept chilled at -20°C until further processing.

2. Slide Preparation and Staining: Labelled grease-free glass slides were prepared with specimen details. A few drops of the cell suspension were dropped onto the slides from a height of approximately 1.5 feet. Slides were air-dried and gently heated to fix the chromosomes. After proper air-drying, slides were flooded with Giemsa stain and allowed to stand for 10-15 minutes. Slides were then washed with running tap water and air-dried.

3. Microscopic Examination and Analysis: Slides were observed under a microscope at 40X and 100X magnification to visualize chromosomes. Chromosomes were examined for number, morphology, and any abnormalities. Karyotypes were prepared by analyzing and documenting the characteristics of chromosomes for each fish species. Chromosome number and morphology were recorded, and any anomalies were noted. The microscope used for examination was [Infinity] , the camera which was used was “Micaps ECOCMOS510B S/N:C 2312080617” along with Micaps Software.

4. Data Analysis: Data obtained from karyotyping, including chromosome number and morphology, were analyzed and a Karyogram was prepared. Results were documented and compared to existing literature for validation. Interpretation of karyotypes was performed to understand the genetic characteristics of the three fish species under study.

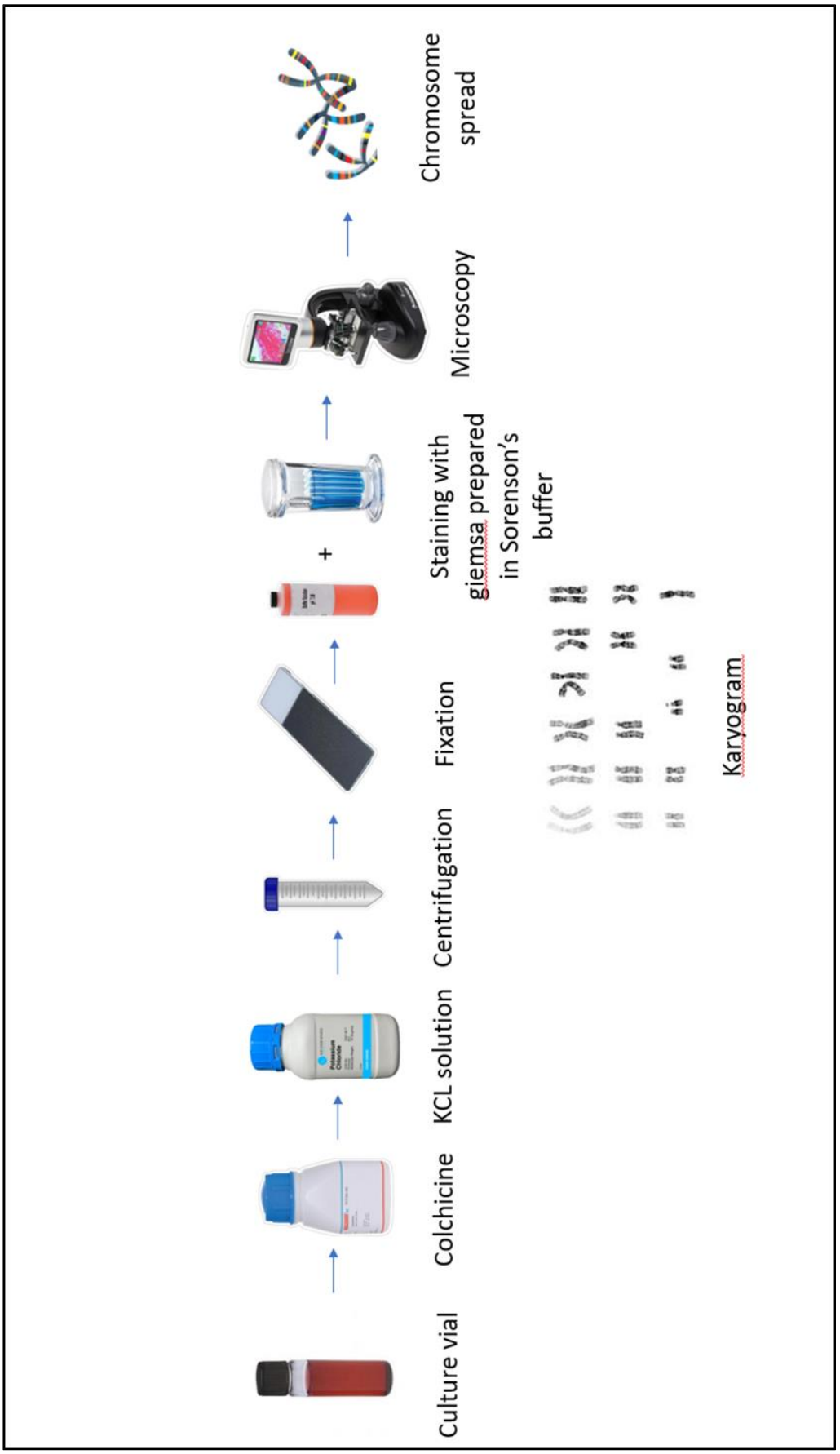


Figure 5- Flowchart of Protocol for Karyotyping

3.1.6. Protocol for Basic Chromosomal Characterization:

1. Microscopic Analysis and Image Capture: Metaphase spreads of chromosomes were prepared from cultured blood cells on glass slides. The slides were placed under a microscope equipped with an image analyzer. High-resolution images of at least five good metaphase spreads from each species were captured.

2. **Prints and Karyogram Preparation: T**he captured images of metaphase spreads were printed. Karyograms were arranged by organizing chromosomes from largest to smallest based on size. Chromosomal morphology and chromosome enumeration were analyzed using the karyograms.

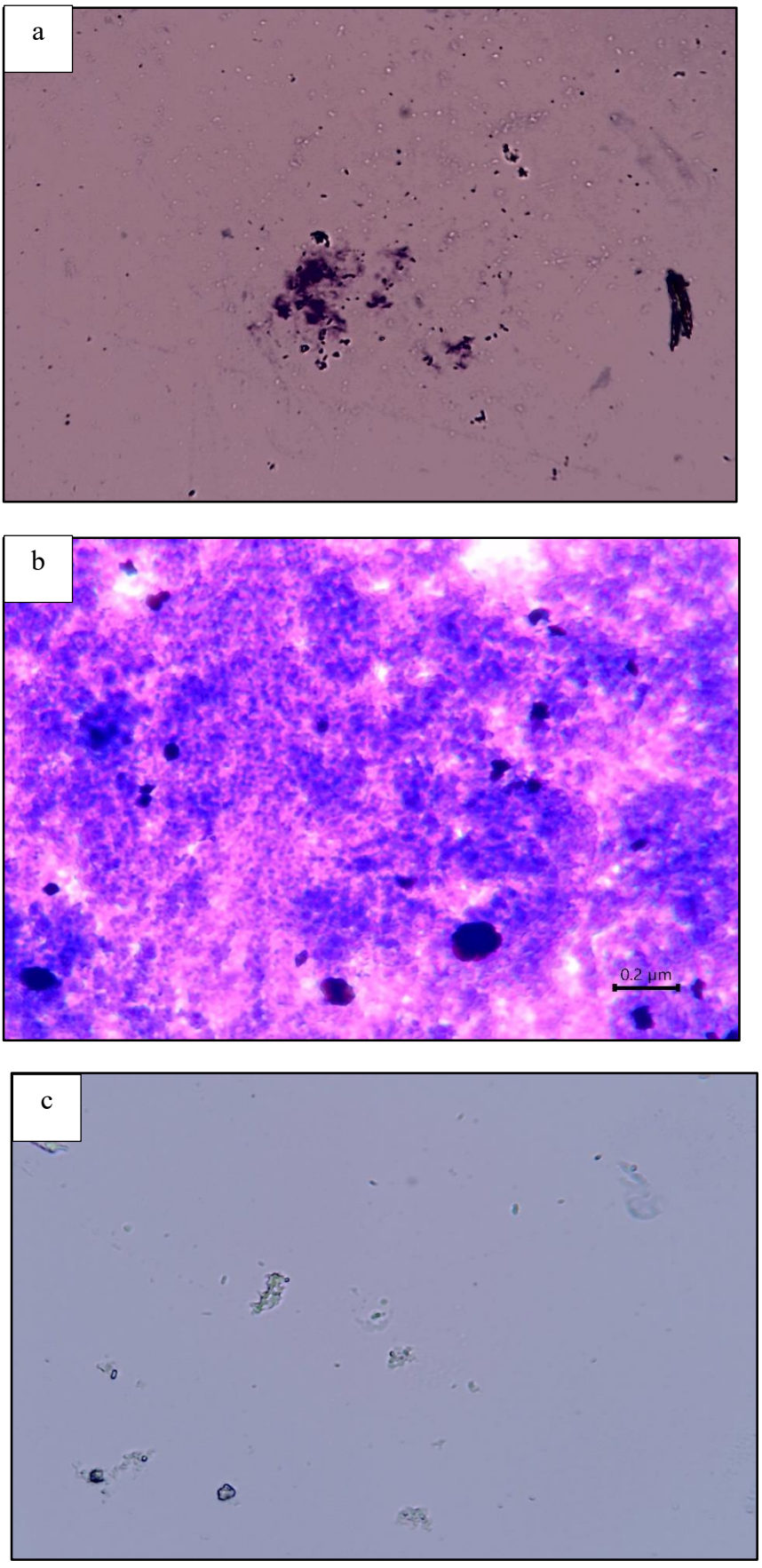
3. **Data Authenticity:** The authenticity of the data was ensured by using metaphase spreads from five different specimens of each fish species. Chromosomal features observed across multiple specimens were compared and validated.

4. **Analysis and Interpretation:** The karyograms were analyzed to determine the chromosome number and morphology of each fish species. Any variations or similarities observed in chromosomal features among the species were recorded and documented. The findings were interpreted to characterize the chromosomal features of Indian Mackerel, Indian Oil Sardine, and Grey Mullet.

5 Data Presentation: The analyzed data, including karyograms and interpretations, were presented in a clear and organized manner. The protocol and data analysis methods were reviewed to ensure consistency and reliability.

Following this protocol, the chromosomal features of Indian Mackerel, Indian Oil Sardine, and Grey Mullet were accurately characterized through karyotype analysis.

Figure 6- Staining: a) Perfectly stained slide b) Over-stained slide and c) Under-stained slide



CHAPTER 4

ANALYSIS AND CONCLUSION

4 .ANALYSIS AND CONCLUSION

4.1 ANALYSIS

Table 2. presents the effect of different incubation temperatures on fish blood cell cultures from Indian Mackerel established using RPMI medium supplemented with FBS. The data is represented as Mean ± Standard deviation for n= 3 replicates. Statistical analysis using one-way ANOVA revealed a significant difference between the various temperature groups ($p<0.0001$, $F=119$). On Day 1, the cell counts were relatively consistent at 20°C and 27°C, with mean values of 950.00 ± 50.00 and 950.00 ± 50.00, respectively. At 25°C and 30°C, the cell counts were higher, with mean values of 1033.33 ± 57.74 and 1033.33 ± 57.74, respectively. By Day 2, a notable decrease in cell counts was observed at 25°C, where the mean count dropped to 0.00 ± 0.00. In contrast, cultures at 20°C, 27°C, and 30°C maintained relatively stable cell counts, with mean values of 516.67 ± 104.08, 483.33 ± 76.38, and 516.67 ± 125.83, respectively. On Day 3, cultures incubated at 20°C and 27°C showed no cell proliferation, with mean counts remaining at 0.00. However, at 27°C, there was a significant increase in cell count compared to the previous day, with a mean value of 116.67 ± 76.38. Cultures at 30°C also demonstrated an increase in cell count on Day 3, with a mean value of 233.33 ± 57.74.

These results indicate temperature-dependent effects on fish blood cell culture proliferation, with optimal growth observed at 25°C on Day 1 and at 27°C on Day 3 (Figure 7).

Table 2: Effect of different temperatures on fish blood cell culture from Indian Mackerel established with RPMI medium supplemented with FBS. Values shown are Mean ± Standard deviation for n= 3. One-way ANOVA between various temperature groups is significant at p<0.0001 (11, F=119)

	Incubating temperatures			
	20ºC	**25ºC**	**27ºC**	**30ºC**
Day 1	950.00 ± 50.00	1033.33 ±57.74	950.00 ±50.00	1033.33 ±57.74
Day 2	516.67 ±104.08	0.00 ±0.00	483.33 ±76.38	516.67 ±125.83
Day 3	0.00	0.00	116.67 ±76.38	233.33 ±57.74

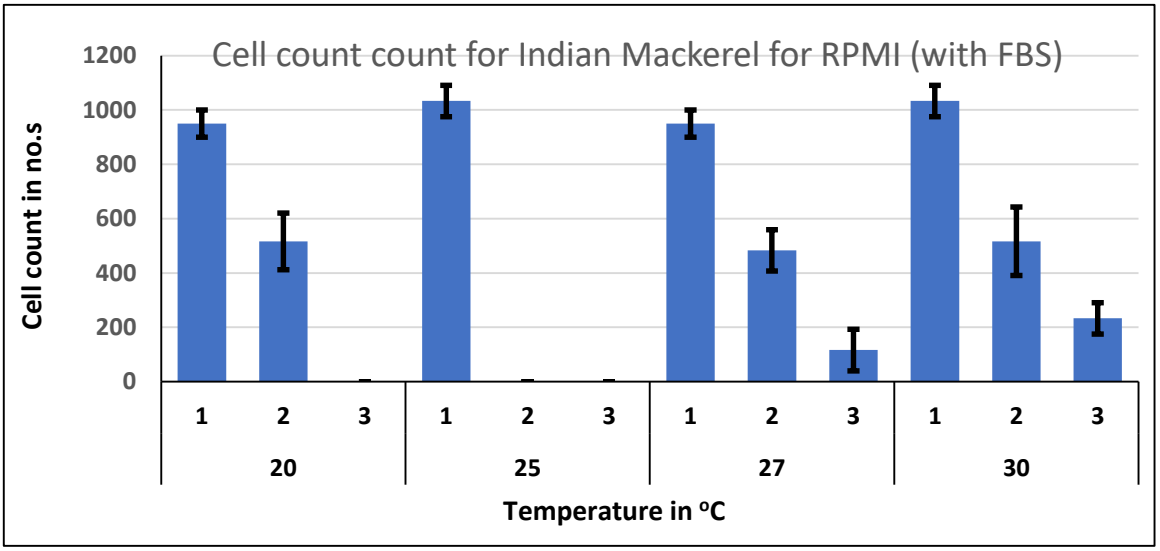


Figure 7: Effect of different temperatures on fish blood cell culture from Indian Mackerel established with RPMI medium supplemented with FBS. Values shown are Mean ± Standard deviation for n= 3. One-way ANOVA between various temperature groups is significant at p<0.0001 (11, F=119)

Table 3 presents the effect of different incubation temperatures on fish blood cell cultures from Indian Mackerel established using DMEM medium supplemented with FBS. The data is presented as Mean ± Standard deviation for n= 3 replicates. Statistical analysis using one-way ANOVA revealed a significant difference between the various temperature groups ($p<0.0001$, $F=533$). On Day 1, the cell counts varied across different temperatures. The highest mean cell count was observed at 20°C, with a value of 1033.33 ± 57.74, followed by 27°C with 983.33 ± 28.87, and 30°C with 1016.67 ± 28.87. At 25°C, the mean cell count was slightly lower at 950.00 ± 50.00. By Day 2, a decrease in cell counts was observed across all temperatures compared to Day 1. At 20°C and 25°C, the mean cell counts decreased to 450.00 ± 50.00. At 27°C, the mean cell count was 666.67 ± 28.87. Interestingly, at 30°C, the cell count dropped to 0.00, indicating a complete cessation of cell proliferation. On Day 3, no viable cells were observed across all temperatures, with mean cell counts of 0.00.

These results suggest that the DMEM medium supplemented with FBS supports initial cell proliferation, with optimal growth observed at 20°C on Day 1. However, cell proliferation decreases rapidly in subsequent days, with no viable cells observed beyond Day 2 (Figure 8).

Table 3 : Effect of different temperatures on fish blood cell culture from Indian Mackerel established with DMEM medium supplemented with FBS. Values shown are Mean ± Standard deviation for n= 3. One-way ANOVA between various temperature groups is significant at p<0.0001 (11, F=533)

	Incubating temperatures			
	20ºC	**25ºC**	**27ºC**	**30ºC**
Day 1	1033.33 ± 57.74	950.00 ±50.00	983.33 ±28.87	1016.67 ±28.87
Day 2	450.00 ±50.00	450.00 ±50.00	666.67 ±28.87	0.00
Day 3	0.00	0.00	0.00	0.00

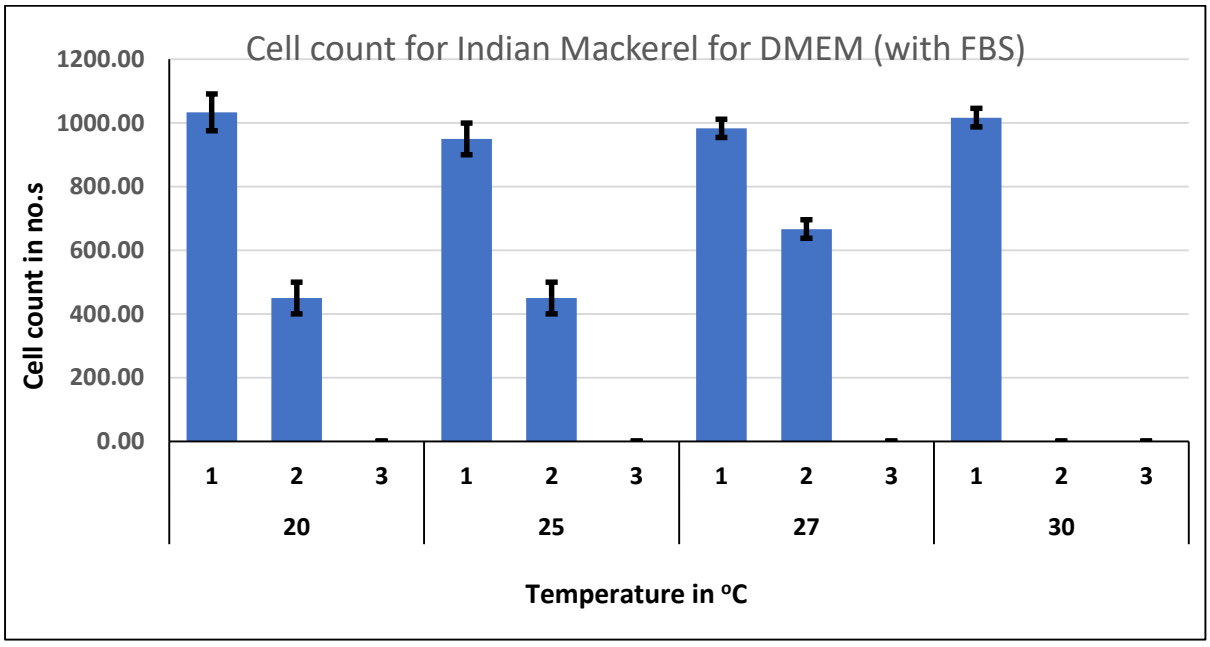


Figure 8: Effect of different temperatures on fish blood cell culture from Indian Mackerel established with DMEM medium supplemented with FBS. Values shown are Mean ± Standard deviation for n= 3. One-way ANOVA between various temperature groups is significant at p<0.0001 (11, F=533).

Table 4 illustrates the impact of different incubation temperatures on fish blood cell cultures from Indian Mackerel established with McCoy's medium supplemented with FBS. The data is presented as Mean ± Standard deviation for n= 3 replicates. Statistical analysis using one-way ANOVA indicates a significant difference between the various temperature groups ($p<0.0001$, $F=1484$). On Day 1, cell counts varied slightly across different temperatures. The highest mean cell count was observed at 25°C, with a value of 1033.33 ± 57.74, followed by 20°C and 27°C, both with a mean cell count of 983.33 ± 28.87. At 30°C, the mean cell count remained consistent with the other temperatures at 983.33 ± 28.87. By Day 2, no viable cells were observed across all temperatures, indicated by a mean cell count of 0.00. On Day 3, consistent with Day 2, no viable cells were detected across any of the temperatures, with mean cell counts remaining at 0.00.

These results suggest that McCoy's medium supplemented with FBS is not conducive to sustaining cell proliferation beyond Day 1 under the tested temperature conditions (Figure 9).

Table 4: Effect of different temperatures on fish blood cell culture from Indian Mackerel established with Mc'coys medium supplemented with FBS. Values shown are Mean ± Standard deviation for n= 3. One-way ANOVA between various temperature groups is significant at p<0.0001 (11, F=1484.)

	Incubating temperatures			
	20ºC	**25ºC**	**27ºC**	**30ºC**
Day 1	983.33 ± 28.87	1033.33 ±57.74	983.33 ±28.87	983.33 ±28.87
Day 2	0.00	0.00	0.00	0.00
Day 3	0.00	0.00	0.00	0.00

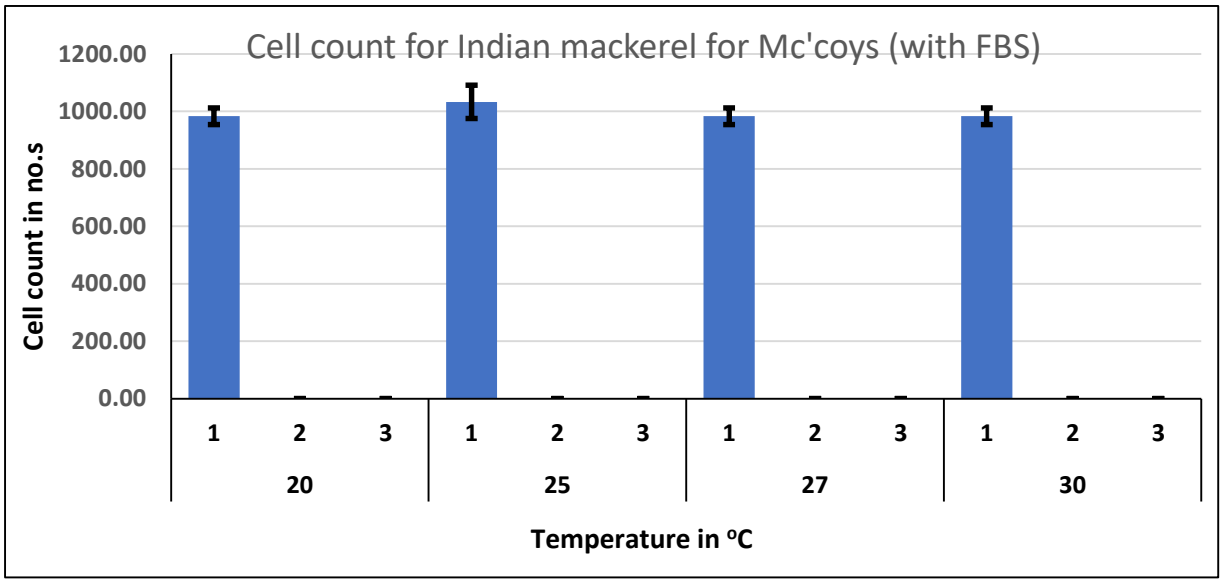


Figure 9: Effect of different temperatures on fish blood cell culture from Indian Mackerel established with RPMI medium supplemented with FBS. Values shown are Mean ± Standard deviation for n= 3. One-way ANOVA between various temperature groups is significant at p<0.0001 (11, F=1484)

Table 5 displays the influence of different incubation temperatures on fish blood cell cultures from Flathead Grey Mullet established with RPMI medium supplemented with FBS. The data is presented as Mean ± Standard deviation for n= 3 replicates. Statistical analysis using one-way ANOVA indicates a significant difference between the various temperature groups ($p<0.0001$, $F=532$). On Day 1, varying cell counts were observed across different temperatures. The highest mean cell count was recorded at 27°C, with a value of 1200.00 ± 50.00, followed by 25°C with a mean cell count of 1066.67 ± 57.74. At 20°C, the mean cell count was 983.33 ± 28.87, while at 30°C, it was 883.33 ± 28.87. By Day 2, a decline in cell counts was observed across all temperatures compared to Day 1. At 25°C and 20°C, mean cell counts decreased to 900.00 ± 0.00 and 850.00 ± 50.00, respectively. However, at 27°C, the mean cell count remained relatively stable at 850.00 ± 50.00. At 30°C, a sharp decline in cell count was observed to 183.33 ± 28.87. On Day 3, no viable cells were detected at 20°C and 25°C, with mean cell counts remaining at 0.00. At 27°C, a decrease in cell count was observed compared to Day 2, with a mean cell count of 416.67 ± 28.87. Similar to the previous days, no viable cells were observed at 30°C, with a mean cell count of zero.

These findings suggest that the optimal temperature for maintaining Flathead Grey Mullet blood cell cultures varies between 25°C and 27°C, with higher temperatures potentially impacting cell viability and proliferation (Figure 10).

Table 5: Effect of different temperatures on fish blood cell culture from Flathead Grey Mullet established with RPMI medium supplemented with FBS. Values shown are Mean ± Standard deviation for n= 3. One-way ANOVA between various temperature groups is significant at p<0.0001 (11, F=532).

	Incubating temperatures			
	20ºC	**25ºC**	**27ºC**	**30ºC**
Day 1	983.33 ± 28.87	1066.67 ±57.74	1200.00 ±50.00	883.33 ±28.87
Day 2	850.00 ± 50.00	900.00 ± 0.00	850.00 ±50.00	183.33 ±28.87
Day 3	0.00	0.00	416.67 ±28.87	0.00

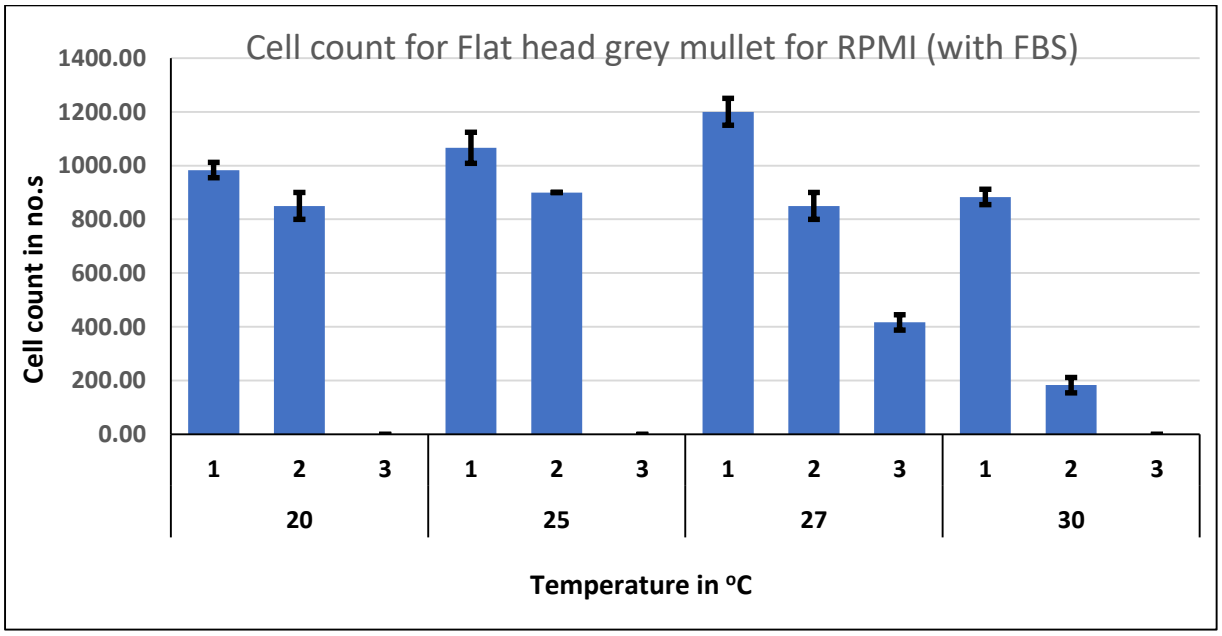


Figure 10: Effect of different temperatures on fish blood cell culture from Indian Mackerel established with RPMI medium supplemented with FBS. Values shown are Mean ± Standard deviation for n= 3. One-way ANOVA between various temperature groups is significant at p<0.0001 (11, F=532).

Table 6: illustrates the impact of different incubation temperatures on fish blood cell cultures from Flathead Grey Mullet established with DMEM medium supplemented with FBS. The data is presented as Mean ± Standard deviation for n= 3 replicates. Statistical analysis using one-way ANOVA reveals a significant difference between the various temperature groups ($p<0.0001$, $F=333$). On Day 1, varying cell counts were observed across different temperatures. The highest mean cell count was recorded at 20°C, with a value of 1183.33 ± 76.38, followed by 27°C with a mean cell count of 1050.00 ± 50.00. At 25°C, the mean cell count was 1033.33 ± 104.08, while at 30°C, it was 1016.67 ± 28.87. By Day 2, a decline in cell counts was observed across all temperatures compared to Day 1. At 20°C, the mean cell count decreased to 1000.00 ± 0.00, while at 27°C and 25°C, it decreased to 616.67 ± 28.87 and 650.00 ± 50.00, respectively. At 30°C, a further decrease in cell count was observed to 116.67 ± 28.87. On Day 3, no viable cells were detected at 20°C and 25°C, with mean cell counts remaining at 0.00. At 27°C, a decrease in cell count was observed compared to Day 2, with a mean cell count of 183.33 ± 28.87. Similarly, no viable cells were observed at 30°C, with a mean cell count of 0.00.

These findings suggest that the optimal temperature for maintaining Flathead Grey Mullet blood cell cultures with DMEM medium varies between 20°C and 27°C, with higher temperatures adversely affecting cell viability and proliferation (Figure 11).

Table 6: Effect of different temperatures on fish blood cell culture from Flathead Grey Mullet established with DMEM medium supplemented with FBS. Values shown are Mean ± Standard deviation for n= 3. One-way ANOVA between various temperature groups is significant at p<0.0001 (11, F= 333)

	Incubating temperatures			
	20ºC	**25ºC**	**27ºC**	**30ºC**
Day 1	1183.33 ± 76.38	1033.33 ±104.08	1050.00 ±50.00	1016.67 ±28.87
Day 2	1000.00 ± 0.00	650.00 ± 50.00	616.67 ±28.87	116.67 ±28.87
Day 3	0.00	0.00	183.33 ±28.87	0.00

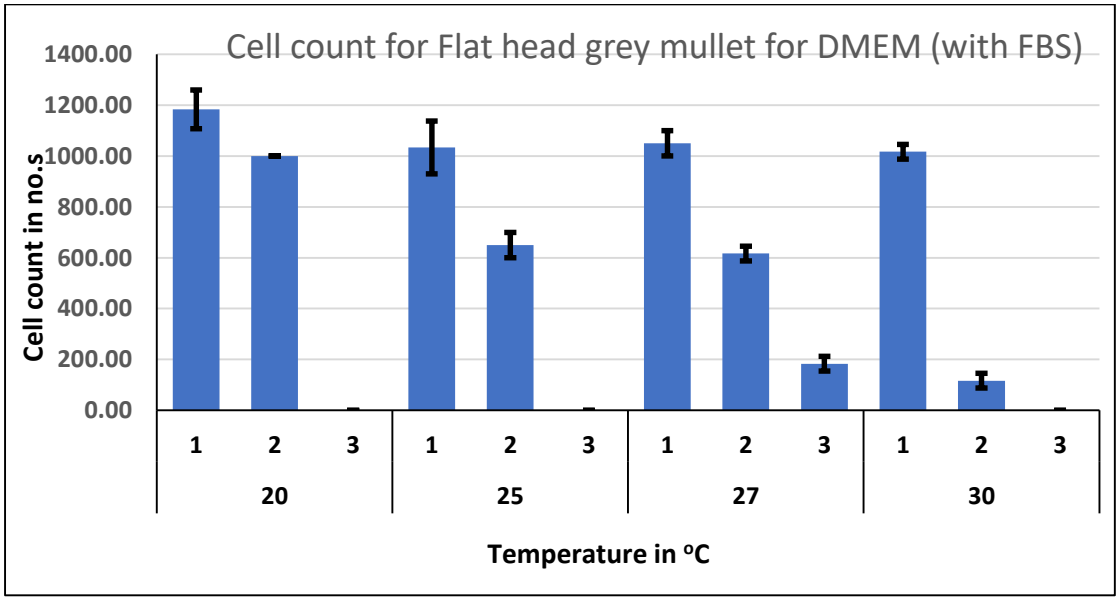


Figure 11: Effect of different temperatures on fish blood cell culture from Flathead Grey Mullet established with DMEM medium supplemented with FBS. Values shown are Mean ± Standard deviation for n= 3. One-way ANOVA between various temperature groups is significant at p<0.0001 (11, F= 333)

Table 7 presents the effects of different incubation temperatures on fish blood cell cultures from Flathead Grey Mullet established with McCoy's medium supplemented with FBS. The data is represented as Mean ± Standard deviation for n= 3 replicates, with statistical significance determined by one-way ANOVA ($p<0.0001$, $F=580$). On Day 1, consistent cell counts were observed across all temperatures, with a mean cell count of 1050.00 ± 50.00 at 20°C, 1050.00 ± 86.60 at 25°C, 1050.00 ± 50.00 at 27°C, and 1050.00 ± 50.00 at 30°C. By Day 2, a significant decrease in cell counts was observed at 20°C and 27°C, with no viable cells detected. At 25°C, a mean cell count of 416.67 ± 28.87 was observed, indicating a decline in cell viability compared to Day 1. Similarly, no viable cells were observed at 30°C. On Day 3, no viable cells were detected across all temperatures, with mean cell counts remaining at 0.00.

These results suggest that McCoy's medium supplemented with FBS may not be suitable for maintaining Flathead Grey Mullet blood cell cultures under the tested temperature conditions (figure 12).

Table 7: Effect of different temperatures on fish blood cell culture from Flathead Grey Mullet established with Mc' coys medium supplemented with FBS. Values shown are Mean ± Standard deviation for n= 3. One-way ANOVA between various temperature groups is significant at p<0.0001 (11, F=580)

	Incubating temperatures			
	20ºC	**25ºC**	**27ºC**	**30ºC**
Day 1	1050.00 ± 50.00	1050.00 ±86.60	1050.00 ±50.00	1050.00 ±50.00
Day 2	0.00	416.67 ± 28.87	0.00	00.00
Day 3	0.00	0.00	0.00	0.00

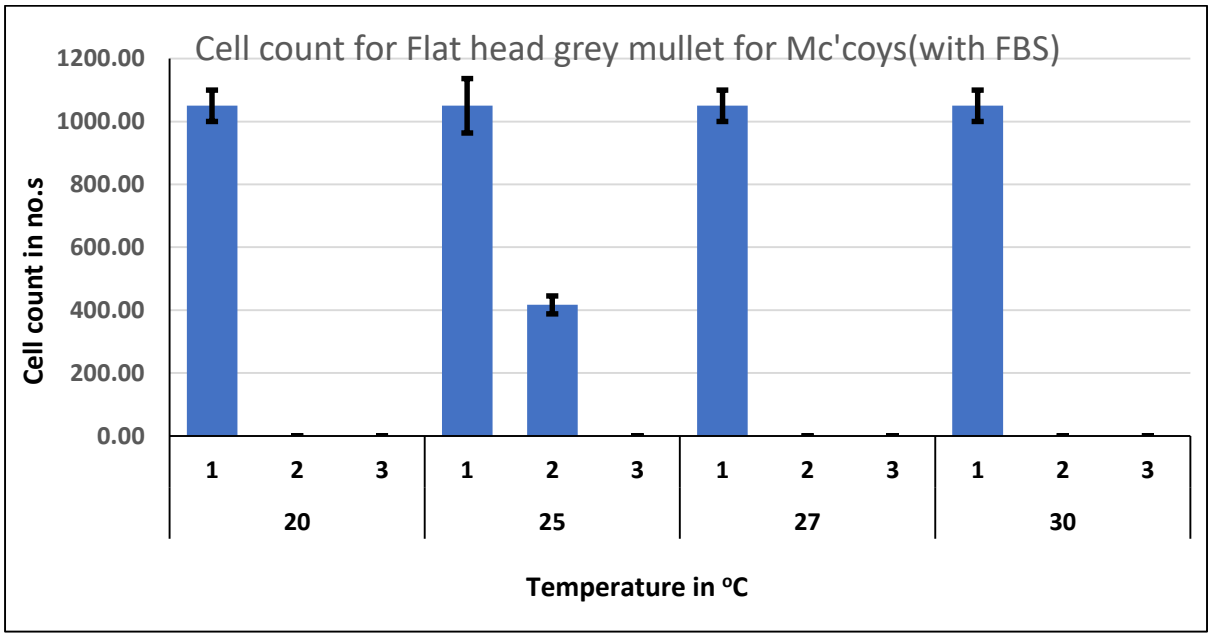


Figure 12: Effect of different temperatures on fish blood cell culture from Flathead Grey Mullet established with Mc' coys medium supplemented with FBS. Values shown are Mean ± Standard deviation for n= 3. One-way ANOVA between various temperature groups is significant at p<0.0001 (11, F=580)

Table 8 displays the effects of different incubation temperatures on fish blood cell cultures from Indian Oil Sardine established with RPMI medium supplemented with FBS. The data is presented as Mean ± Standard deviation for n= 3 replicates, with one-way ANOVA indicating statistical significance at $p<0.0001$ ($F=157$, $df=11$). On Day 1, varying cell counts were observed across different temperatures. At 20°C, the mean cell count was 833.33 ± 28.87, while at 25°C and 27°C, it was 816.67 ± 28.87 and 883.33 ± 28.87, respectively. The highest cell count was recorded at 30°C, with a mean of 1016.67 ± 28.87. By Day 2, a decrease in cell counts was observed across all temperatures. At 20°C, the mean cell count decreased to 600.00 ± 0.00, while at 25°C, it was 416.67 ± 28.87. At 27°C and 30°C, the mean cell counts were 583.33 ± 76.38 and 300.00 ± 50.00, respectively. On Day 3, further reductions in cell counts were noted. At 20°C, 25°C, and 27°C, the mean cell counts were 216.67 ± 28.87, 183.33 ± 28.87, and 400.00 ± 50.00, respectively. At 30°C, the mean cell count decreased to 250.00 ± 50.00.

These findings suggest that incubation temperature has a significant impact on the viability and proliferation of Indian Oil Sardine blood cell cultures (Figure 13).

Table 8: Effect of different temperatures on fish blood cell culture from Indian Oil Sardine established with RPMI medium supplemented with FBS. Values shown are Mean ± Standard deviation for n= 3. . One-way ANOVA between various temperature groups is significant at p<0.0001 (11, F= 157).

	Incubating temperatures			
	20ºC	**25ºC**	**27ºC**	**30ºC**
Day 1	833.33 ± 28.87	816.67 ±28.87	883.33 ±28.87	1016.67 ±28.87
Day 2	600.00 ± 00.00	416.67 ± 28.87	583.33 ± 76.38	300.00 ±50.00
Day 3	216.67 ± 28.87	183.33 ± 28.87	400.00 ± 50.00	250.00 ±50.00

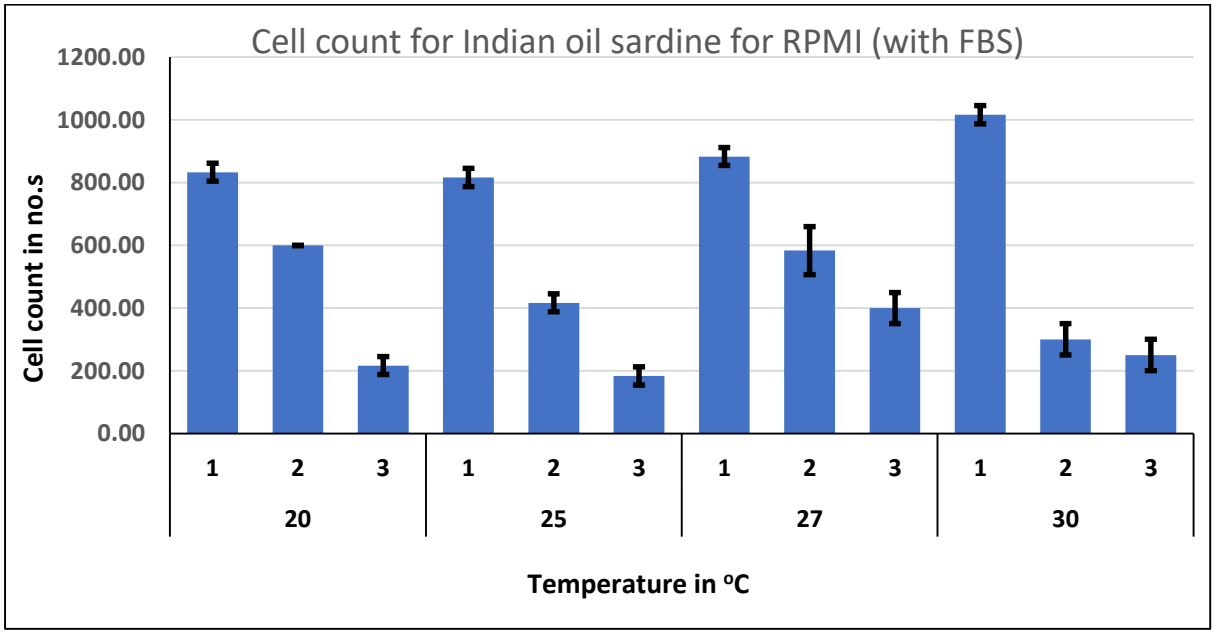


Figure 13: Effect of different temperatures on fish blood cell culture from Indian Oil Sardine established with RPMI medium supplemented with FBS. Values shown are Mean ± Standard deviation for n= 3. One-way ANOVA between various temperature groups is significant at p<0.0001 (11, F= 157).

Table 9 illustrates the impact of different incubation temperatures on fish blood cell cultures from Indian Oil Sardine, established with DMEM medium supplemented with FBS. The data is presented as Mean ± Standard deviation for n= 3 replicates, with one-way ANOVA indicating statistical significance at $p<0.0001$ ($F=108$, $df=11$). On Day 1, varying cell counts were observed at different temperatures. At 20°C, the mean cell count was 950.00 ± 50.00, while at 25°C, it decreased to 816.67 ± 28.87. At 27°C, the mean cell count increased to 900.00 ± 100.00, and at 30°C, it decreased again to 833.33 ± 28.87. By Day 2, further fluctuations in cell counts were observed. At 20°C, the mean cell count decreased to 450.00 ± 50.00, while at 25°C, it increased to 600.00 ± 50.00. At 27°C, the mean cell count was 550.00 ± 50.00, and at 30°C, it decreased to 400.00 ± 50.00. On Day 3, varying trends in cell counts continued. At 20°C and 30°C, no cells were detected (0.00), while at 25°C, the mean cell count was 200.00 ± 50.00. At 27°C, the mean cell count increased to 433.33 ± 28.87.

These results suggest that incubation temperature significantly influences the proliferation and viability of Indian Oil Sardine blood cell cultures, highlighting the importance of temperature optimization for cell culture studies (Figure 14).

Table 9: Effect of different temperatures on fish blood cell culture from Indian Oil Sardine established with DMEM medium supplemented with FBS. Values shown are Mean ± Standard deviation for n= 3. One-way ANOVA between various temperature groups is significant at p<0.0001 (11, F= 108)

	Incubating temperatures			
	20ºC	**25ºC**	**27ºC**	**30ºC**
Day 1	950.00 ± 50.00	816.67 ±28.87	900.00 ±100.00	833.33 ±28.87
Day 2	450.00 ± 50.00	600.00 ± 50.00	550.00 ± 50.00	400.00 ±50.00
Day 3	00.00	200.00 ± 50.00	433.33 ± 28.87	250.00 ±50.00

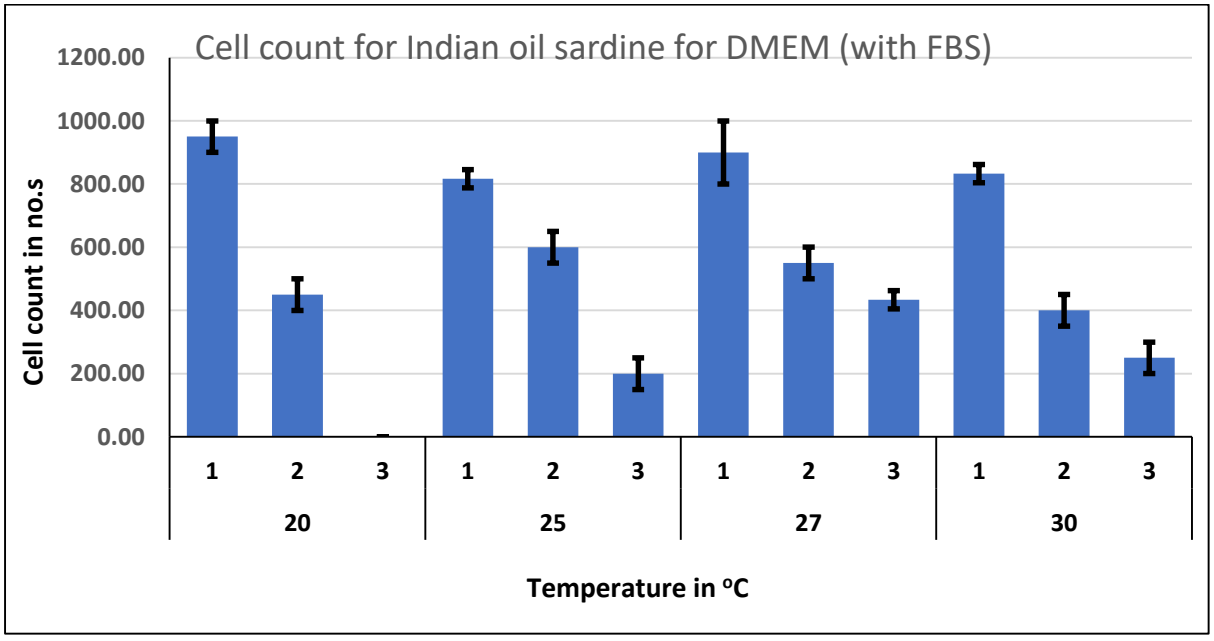


Figure 14: Effect of different temperatures on fish blood cell culture from Indian Oil Sardine established with DMEM medium supplemented with FBS. Values shown are Mean ± Standard deviation for n= 3. One-way ANOVA between various temperature groups is significant at p<0.0001 (11, F= 108)

Table 10 presents the effect of different incubation temperatures on fish blood cell culture from Indian Oil Sardine, established with McCoy's medium supplemented with FBS. The data is depicted as Mean ± Standard deviation for n= 3 replicates, with one-way ANOVA indicating statistical significance at $p<0.0001$ ($F=387$, $df=11$). On Day 1, distinct variations in cell counts were observed across different temperatures. At 20°C, the mean cell count was 900.00 ± 250.00, while at 25°C, it decreased to 550.00 ± 50.00. At 27°C, the mean cell count increased to 816.67 ± 28.87, and at 30°C, it further increased to 950.00 ± 50.00. By Day 2, the cell counts showed further fluctuations. At 20°C, the mean cell count decreased to 250.00 ± 50.00, while at 25°C, it decreased slightly to 416.67 ± 28.87. At 27°C, the mean cell count decreased to 200.00 ± 50.00, and at 30°C, no cells were detected (0.00). On Day 3, minimal to no cell growth was observed across all temperatures. At 20°C, the mean cell count was 166.67 ± 28.87, while at 25°C, no cells were detected (0.00). Similarly, at 27°C and 30°C, no cells were observed (0.00).

These findings suggest that temperature plays a crucial role in the proliferation and viability of Indian Oil Sardine blood cell cultures, with optimal conditions observed at 27°C (Figure 15).

Table 10: Effect of different temperatures on fish blood cell culture from Indian Oil Sardine established with Mc' coys medium supplemented with FBS. Values shown are Mean ± Standard deviation for n= 3. One-way ANOVA between various temperature groups is significant at p<0.0001 (11, F= 387)

	Incubating temperatures			
	20ºC	**25ºC**	**27ºC**	**30ºC**
Day 1	900.00 ± 250.00	550.00 ±50.00	816.67 ±28.87	950.00 ±50.00
Day 2	250.00 ± 50.00	416.67 ± 28.87	200.00 ± 50.00	00.00
Day 3	166.67 ± 28.87	00.00	00.00	00.00

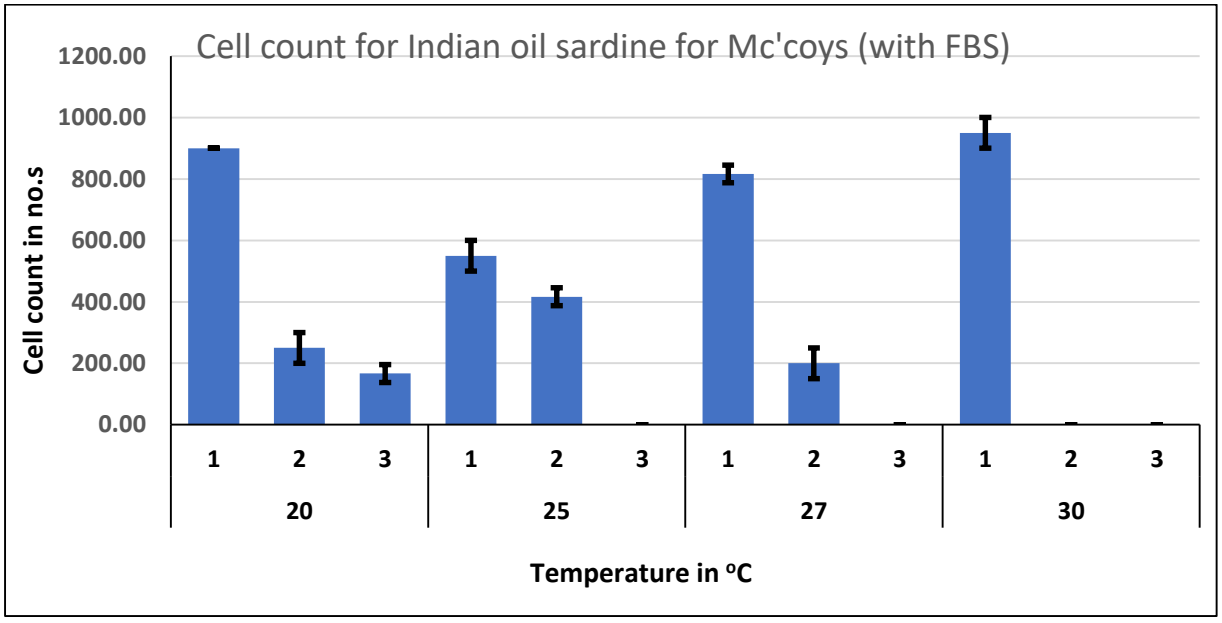


Figure 15: Effect of different temperatures on fish blood cell culture from Vestablished with Mc' coys medium supplemented with FBS. Values shown are Mean ± Standard deviation for n= 3. One-way ANOVA between various temperature groups is significant at p<0.0001 (11, F= 387.)

Table 11 illustrates the impact of different incubation temperatures on fish blood cell culture from Indian Mackerel, established with RPMI medium without FBS supplementation. The data is presented as Mean ± Standard deviation for n= 3 replicates, with one-way ANOVA indicating statistical significance at $p<0.0001$ ($F=125$, $df=11$). On Day 1, varying cell counts were observed across different temperatures. At 20°C, the mean cell count was 1066.67 ± 57.74, while at 25°C, it remained consistent at 1066.67 ± 57.74. At 27°C, a slight decrease in cell count was noted, with a mean of 1033.33 ± 57.74, while at 30°C, there was an increase in cell count to 1133.33 ± 115.47. By Day 2, fluctuations in cell counts were observed. At 20°C and 25°C, the mean cell count remained consistent at 450.00 ± 50.00. At 27°C, there was an increase in cell count to 616.67 ± 28.87, while at 30°C, the count decreased to 500.00 ± 50.00. On Day 3, minimal to moderate cell growth was observed across different temperatures. At 20°C, no cells were detected (0.00), while at 25°C, a mean cell count of 150.00 ± 50.00 was recorded. At 27°C, the cell count increased to 350.00 ± 50.00, and at 30°C, it decreased to 150.00 ± 50.00.

These findings indicate that temperature variation significantly influences cell proliferation in Indian Mackerel blood cell cultures established with RPMI medium without FBS (Figure 16).

Table 11: Effect of different temperatures on fish blood cell culture from Indian Mackerel established with RPMI medium without FBS. Values shown are Mean ± Standard deviation for n= 3. One-way ANOVA between various temperature groups is significant at p<0.0001 (11, F=125)

	Incubating temperatures			
	20ºC	**25ºC**	**27ºC**	**30ºC**
Day 1	1066.67 ± 57.74	1066.67 ±57.74	1033.33 ±57.74	1133.33 ±115.47
Day 2	450.00 ± 50.00	450.00 ± 50.00	616.67 ± 28.87	500.00 ±50.00
Day 3	00.00	150.00 ± 50.00	350.00 ± 50.00	150.00 ±50.00

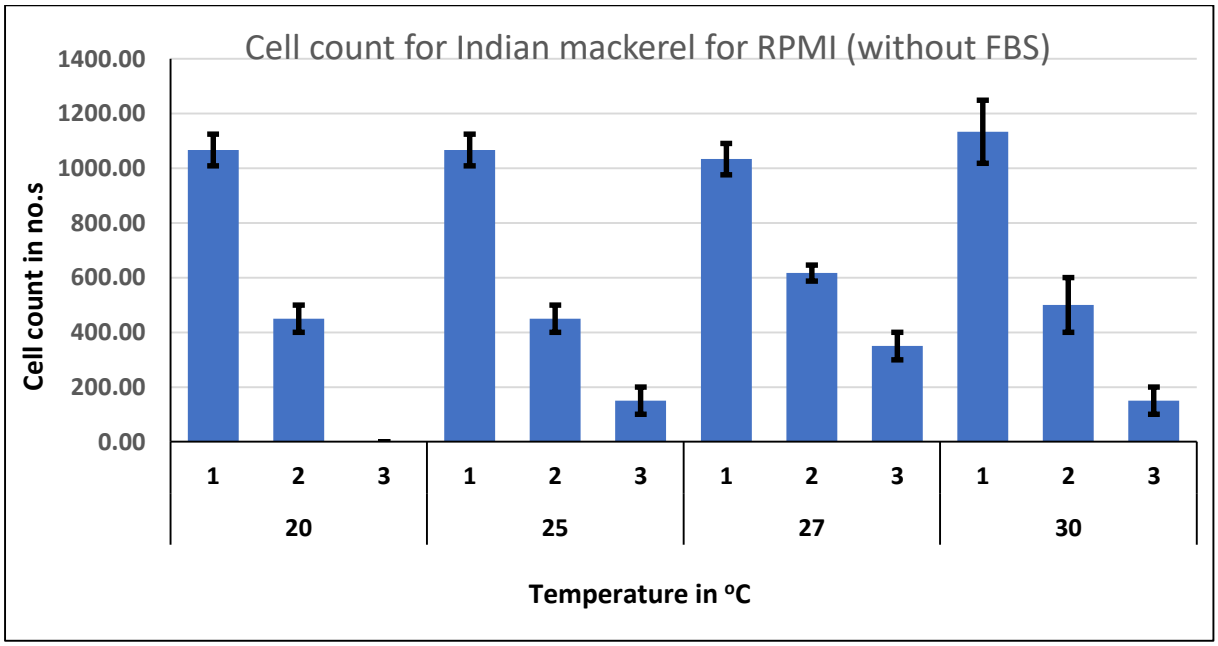


Figure 16: Effect of different temperatures on fish blood cell culture from Indian Mackerel established with RPMI medium without FBS. Values shown are Mean ± Standard deviation for n= 3. One-way ANOVA between various temperature groups is significant at p<0.0001 (11, F=125).

Table 12 presents the effect of different incubation temperatures on fish blood cell culture from Indian Mackerel, established with DMEM medium without FBS supplementation. The data is depicted as Mean ± Standard deviation for n= 3 replicates, with one-way ANOVA indicating statistical significance at $p<0.0001$ ($F= 208$, $df=11$). On Day 1, varying cell counts were observed at different temperatures. At 20°C, the mean cell count was 1050.00 ± 50.00, while at 25°C, it increased to 1133.33 ± 115.47. At 27°C, a slight decrease in cell count was noted, with a mean of 1033.33 ± 57.74, and at 30°C, the count decreased further to 1000.00 ± 50.00.

By Day 2, fluctuations in cell counts were observed. At 20°C, the mean cell count decreased to 183.33 ± 28.87, while at 25°C, it was 150.00 ± 50.00. At 27°C and 30°C, the cell counts increased significantly to 650.00 ± 50.00. On Day 3, varied responses in cell growth were noted. At 20°C and 30°C, no cells were detected (0.00), while at 25°C, a mean cell count of 133.33 ± 28.87 was recorded. At 27°C, the cell count increased substantially to 450.00 ± 50.00.

These findings underscore the significant influence of temperature on cell proliferation in Indian Mackerel blood cell cultures established with DMEM medium without FBS (Figure 17).

Table 12: Effect of different temperatures on fish blood cell culture from Indian Mackerel established with DMEM medium without FBS. Values shown are Mean ± Standard deviation for n= 3. One-way ANOVA between various temperature groups is significant at p<0.0001 (11, F= 208).

	Incubating temperatures			
	20ºC	**25ºC**	**27ºC**	**30ºC**
Day 1	1050.00 ± 50.00	1133.33 ±115.47	1033.33 ±57.74	1000.00 ±50.00
Day 2	183.33 ± 28.87	150.00 ± 50.00	650.00 ± 50.00	650.00 ±50.00
Day 3	00.00	133.33 ± 28.87	450.00 ± 50.00	00.00

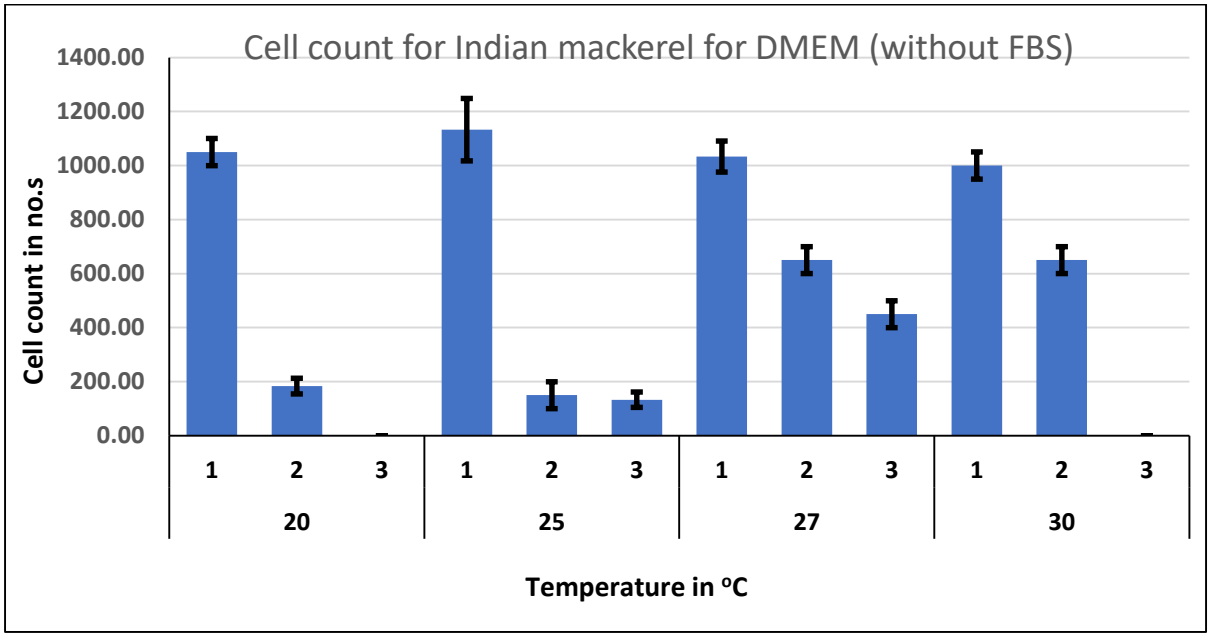


Figure 17: Effect of different temperatures on fish blood cell culture from Indian Mackerel established with DMEM medium with FBS. Values shown are Mean ± Standard deviation for n= 3. One-way ANOVA between various temperature groups is significant at p<0.0001 (11, F= 208).

Table 13 presents the impact of different incubation temperatures on fish blood cell culture from Indian Mackerel, established with McCoy's medium without FBS supplementation. The data is represented as Mean ± Standard deviation for n= 3 replicates, with one-way ANOVA indicating statistical significance at $p<0.0001$ ($F= 208$, $df=11$). On Day 1, varying cell counts were observed at different temperatures. At 20°C, the mean cell count was 1000.00 ± 0.00, while at 25°C, it increased to 1100.00 ± 50.00. At both 27°C and 30°C, the mean cell count remained stable at 1050.00 ± 50.00. By Day 2, significant fluctuations in cell counts were noted. At 20°C, no cells were detected (0.00), while at 25°C, the mean cell count was 450.00 ± 50.00. At 27°C and 30°C, no cells were observed. On Day 3, no cells were detected across all temperature conditions.

These findings indicate that McCoy's medium without FBS supplementation may not support the sustained growth of Indian Mackerel blood cells under the tested conditions (Figure 18).

Table 13: Effect of different temperatures on fish blood cell culture from Indian Mackerel established with Mc'coy medium without FBS. Values shown are Mean ± Standard deviation for n= 3. One-way ANOVA between various temperature groups is significant at p<0.0001 (11, F= 208)

	Incubating temperatures			
	20ºC	**25ºC**	**27ºC**	**30ºC**
Day 1	1000.00 ± 00.00	1100.00 ± 50.00	1050.00 ±50.00	1050.00 ±50.00
Day 2	00.00	450.00 ± 50.00	00.00	00.00
Day 3	00.00	00.00	00.00	00.00

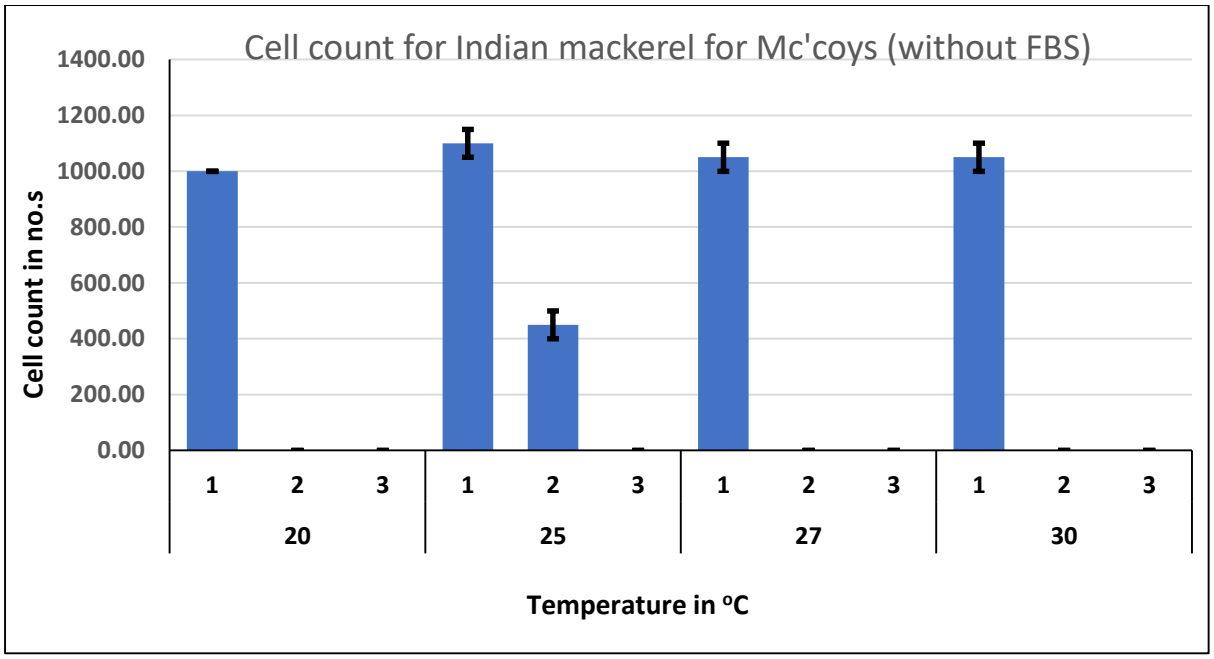


Figure 18: Effect of different temperatures on fish blood cell culture from Indian Mackerel established with DMEM medium without FBS. Values shown are Mean ± Standard deviation for n= 3. One-way ANOVA between various temperature groups is significant at p<0.0001 (11, F= 208).

Table 14 illustrates the impact of different temperatures on fish blood cell culture from Flathead Grey Mullet, established with RPMI medium without FBS supplementation. The data is represented as Mean ± Standard deviation for n= 3 replicates, with one-way ANOVA indicating statistical significance at $p<0.0001$ ($F= 314$, $df=11$). On Day 1, varying cell counts were observed across different temperatures. At 20°C, the mean cell count was 1000.00 ± 50.00, while at 25°C, it increased to 1050.00 ± 50.00. At 27°C, the mean cell count peaked at 1250.00 ± 50.00, and at 30°C, it decreased to 1050.00 ± 50.00. By Day 2, fluctuations in cell counts were noted. At 20°C, the mean cell count was 850.00 ± 50.00, while at 25°C and 27°C, it decreased to 883.33 ± 28.87 and 816.67 ± 28.87, respectively. At 30°C, a significant drop was observed, with a mean cell count of 216.67 ± 28.87. On Day 3, further variations in cell counts were recorded. At 20°C, the mean cell count was 223.33 ± 25.17, while at 25°C, it increased to 241.67 ± 38.19. At 27°C, the mean cell count peaked at 550.00 ± 50.00, whereas at 30°C, no cells were detected (0.00).

These results suggest that RPMI medium without FBS supplementation may support the growth of Flathead Grey Mullet blood cells, with optimal temperatures between 25°C and 27°C (Figure19).

Table 14: Effect of different temperatures on fish blood cell culture from Flathead Grey Mullet established with RPMI medium without FBS. Values shown are Mean ± Standard deviation for n= 3. One-way ANOVA between various temperature groups is significant at p<0.0001 (11, F= 314).

	Incubating temperatures			
	20ºC	**25ºC**	**27ºC**	**30ºC**
Day 1	1000.00 ± 50.00	1050.00 ±50.00	1250.00 ±50.00	1050.00 ±50.00
Day 2	850.00 ± 50.00	883.33 ± 28.87	816.67 ± 28.87	216.67 ±28.87
Day 3	223.33 ± 25.17	241.67 ± 38.19	550.00 ± 50.00	00.00

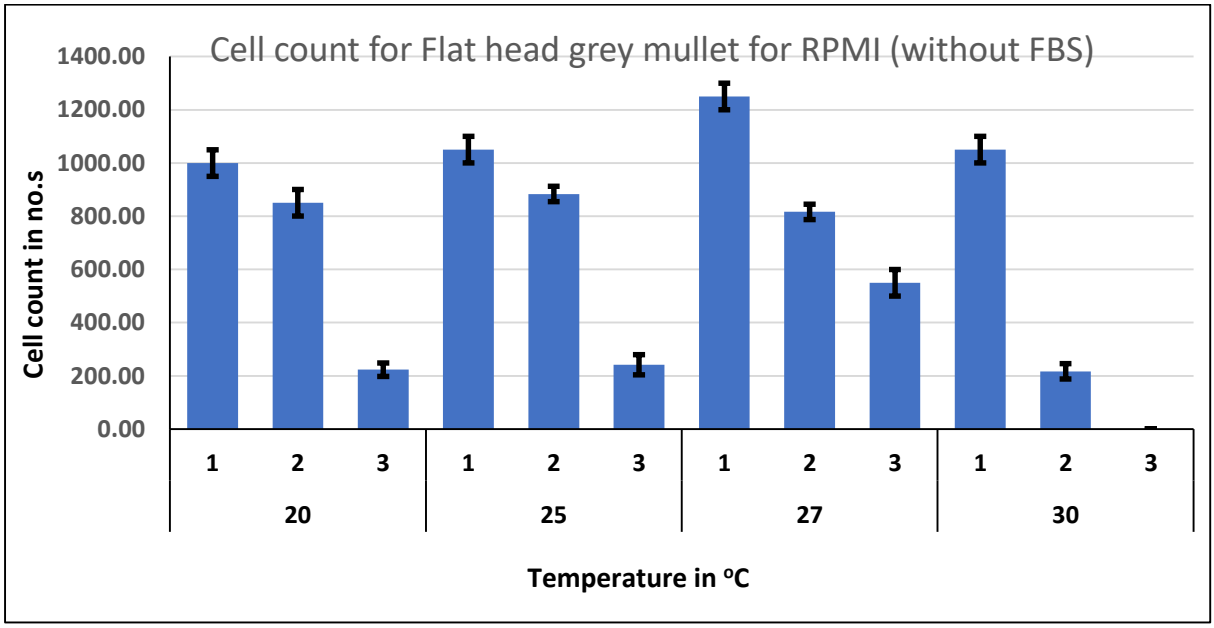


Figure 19: Effect of different temperatures on fish blood cell culture from Flathead Grey Mullet established with RPMI medium without FBS. Values shown are Mean ± Standard deviation for n= 3. One-way ANOVA between various temperature groups is significant at p<0.0001 (11, F= 314).

Table 15 displays the impact of different temperatures on fish blood cell culture from Flathead Grey Mullet, established with DMEM medium without FBS supplementation. The data is presented as Mean ± Standard deviation for n= 3 replicates, with one-way ANOVA indicating statistical significance at $p<0.0001$ ($F= 219$, $df=11$). On Day 1, varying cell counts were observed across different temperatures. At 20°C, the mean cell count was 1083.33 ± 76.38, while at 25°C, it decreased to 950.00 ± 50.00. At 27°C, the mean cell count increased to 1058.33 ± 52.04, and at 30°C, it remained consistent at 1050.00 ± 50.00. By Day 2, fluctuations in cell counts were noted. At 20°C, the mean cell count was 600.00 ± 50.00, while at 25°C, it decreased to 550.00 ± 50.00. At 27°C, a slight increase was observed, with a mean cell count of 816.67 ± 28.87. At 30°C, a significant drop was recorded, with a mean cell count of 250.00 ± 50.00. On Day 3, further variations in cell counts were observed. At 20°C, no cells were detected (0.00), while at 25°C, the mean cell count was 416.67 ± 28.87. At 27°C, a higher mean cell count of 650.00 ± 50.00 was observed, and at 30°C, no cells were detected (0.00).

These results indicate that DMEM medium without FBS supplementation may support the growth of Flathead Grey Mullet blood cells, with optimal temperatures between 27°C and 30°C (Figure20).

Table 15: Effect of different temperatures on fish blood cell culture from Flathead Grey Mullet established with DMEM medium without FBS. Values shown are Mean ± Standard deviation for n= 3. One-way ANOVA between various temperature groups is significant at p<0.0001 (11, F= 219).

	Incubating temperatures			
	20ºC	**25ºC**	**27ºC**	**30ºC**
Day 1	1083.33 ± 76.38	950.00 ±50.00	1058.33 ±52.04	1050.00 ±50.00
Day 2	600.00 ± 50.00	550.00 ± 50.00	816.67 ± 28.87	250.00 ±50.00
Day 3	00.00	416.67 ± 28.87	650.00 ± 50.00	00.00

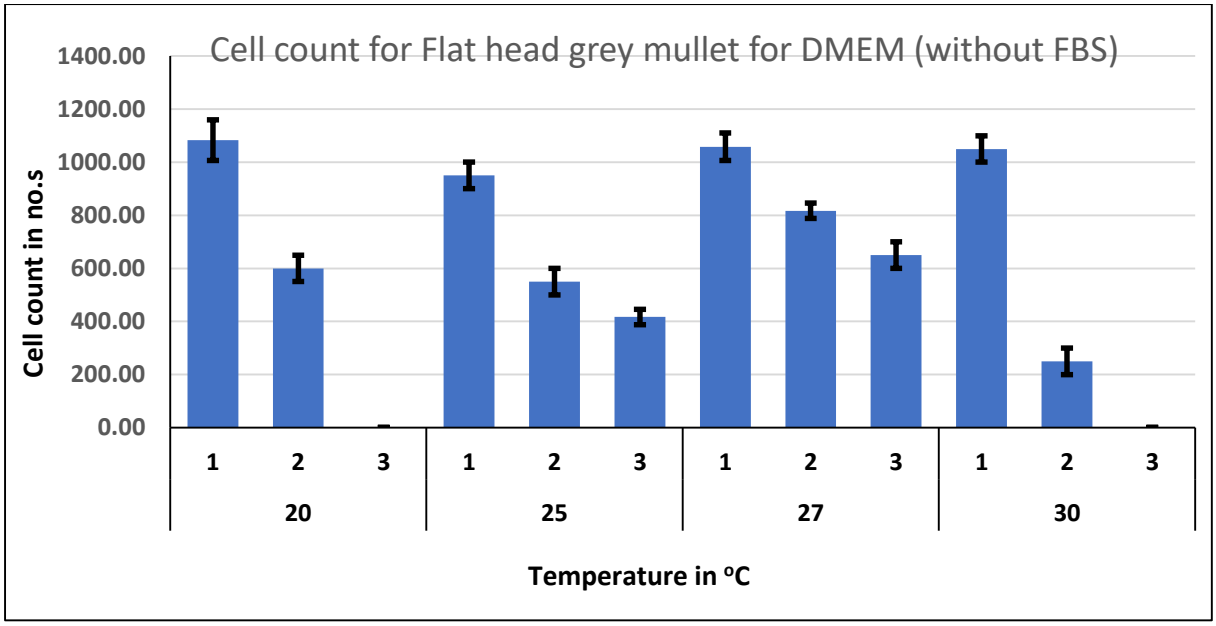


Figure 20: Effect of different temperatures on fish blood cell culture from established with DMEM medium without FBS. Values shown are Mean ± Standard deviation for n= 3. One-way ANOVA between various temperature groups is significant at p<0.0001 (11, F= 219).

Table 16 illustrates the influence of different temperatures on fish blood cell culture from Flathead Grey Mullet, established with McCoy's medium without FBS supplementation. The presented values represent Mean ± Standard deviation for n= 3 replicates, with one-way ANOVA indicating significant differences at $p<0.0001$ ($F= 808$, $df=11$). On Day 1, diverse cell counts were observed across different incubating temperatures. At 20°C, the mean cell count was 1050.00 ± 50.00, while at 25°C, it increased to 1150.00 ± 50.00. Similarly, at 27°C and 30°C, the mean cell counts remained consistent at 1100.00 ± 50.00. By Day 2, no cells were detected at 20°C, while at 25°C, the mean cell count was 450.00 ± 50.00. No cells were observed at 27°C and 30°C. On Day 3, no cells were detected across all incubating temperatures.

These results suggest that McCoy's medium without FBS supplementation may not adequately support the growth and viability of Flathead Grey Mullet blood cells under the tested temperature conditions (Figure 21).

Table 16: Effect of different temperatures on fish blood cell culture from Flathead Grey Mullet established with Mc' coys medium without FBS. Values shown are Mean ± Standard deviation for n= 3. One-way ANOVA between various temperature groups is significant at p<0.0001 (11, F= 808).

	Incubating temperatures			
	20ºC	**25ºC**	**27ºC**	**30ºC**
Day 1	1050.00 ± 50.00	1150.00 ±50.00	1100.00 ±50.00	1100.00 ±50.00
Day 2	00.00	450.00 ± 50.00	00.00	00.00
Day 3	00.00	00.00	00.00	00.00

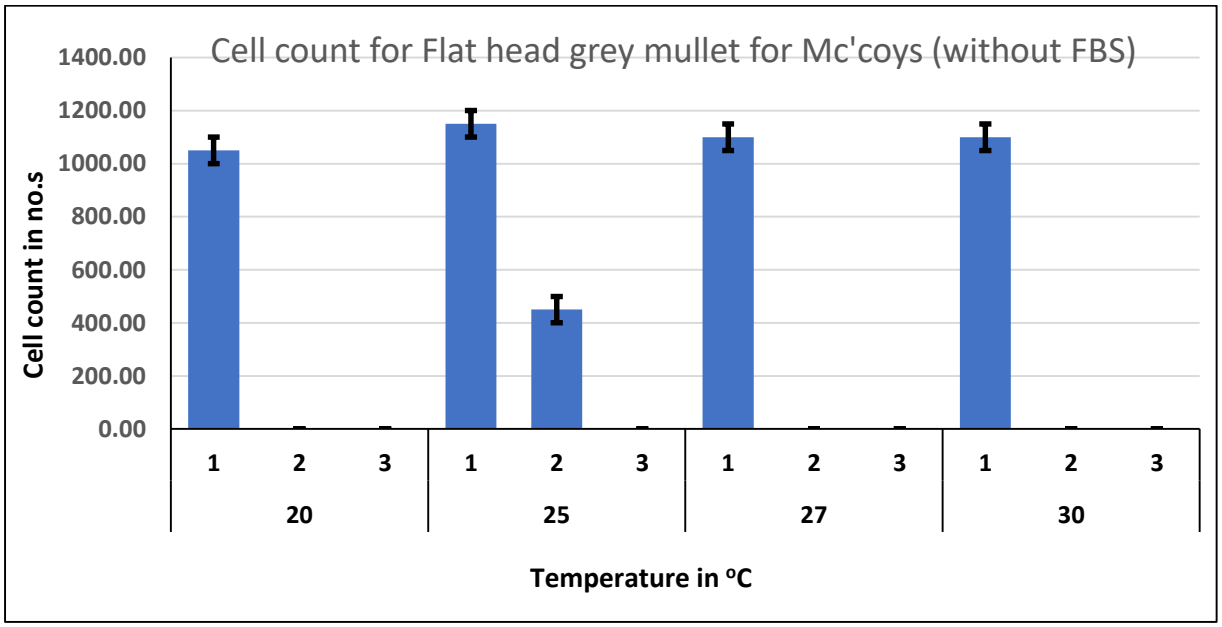


Figure 21: Effect of different temperatures on fish blood cell culture from established with Mc' coys medium with without FBS. Values shown are Mean ± Standard deviation for n= 3. One-way ANOVA between various temperature groups is significant at p<0.0001 (11, F= 808).

Table 17 presents the impact of various temperatures on fish blood cell culture from Indian Oil Sardine, established with RPMI medium without FBS supplementation. The values displayed represent Mean ± Standard deviation for n= 3 replicates, with one-way ANOVA indicating significant differences at $p<0.0001$ ($F=149$, $df=11$). On Day 1, the mean cell counts varied across different incubating temperatures. At 20°C, the mean cell count was 850.00 ± 50.00, while at 25°C, it decreased to 816.67 ± 28.87. The mean cell count remained consistent at 850.00 ± 50.00 for 27°C, and it increased to 1016.67 ± 28.87 at 30°C. By Day 2, a similar trend was observed, with varying mean cell counts. At 20°C, the mean cell count was 650.00 ± 50.00, while at 25°C, it decreased to 400.00 ± 0.00. At 27°C, the mean cell count increased to 700.00 ± 50.00, and it decreased to 350.00 ± 50.00 at 30°C.

On Day 3, the mean cell counts continued to vary across different temperatures. At 20°C, the mean cell count was 216.00 ± 28.87, while at 25°C, it increased to 250.00 ± 50.00. Similarly, at 27°C, the mean cell count was 350.00 ± 50.00, and it decreased to 166.67 ± 28.87 at 30°C.

These findings suggest that RPMI medium without FBS supplementation may influence the growth and viability of Indian Oil Sardine blood cells differently under different temperature conditions (Figure 22).

Table 17: Effect of different temperatures on fish blood cell culture from Indian Oil Sardine established with RPMI medium without FBS. Values shown are Mean ± Standard deviation for n= 3. One-way ANOVA between various temperature groups is significant at p<0.0001 (11, F=149).

	Incubating temperatures			
	20ºC	**25ºC**	**27ºC**	**30ºC**
Day 1	850.00 ± 50.00	816.67 ±28.87	850.00 ±50.00	1016.67 ±28.87
Day 2	650.00 ± 50.00	400.00 ± 00.00	700.00 ± 50.00	350.00 ± 50.00
Day 3	216.00 ± 28.87	250.00 ± 50.00	350.00 ± 50.00	166.67 ± 28.87

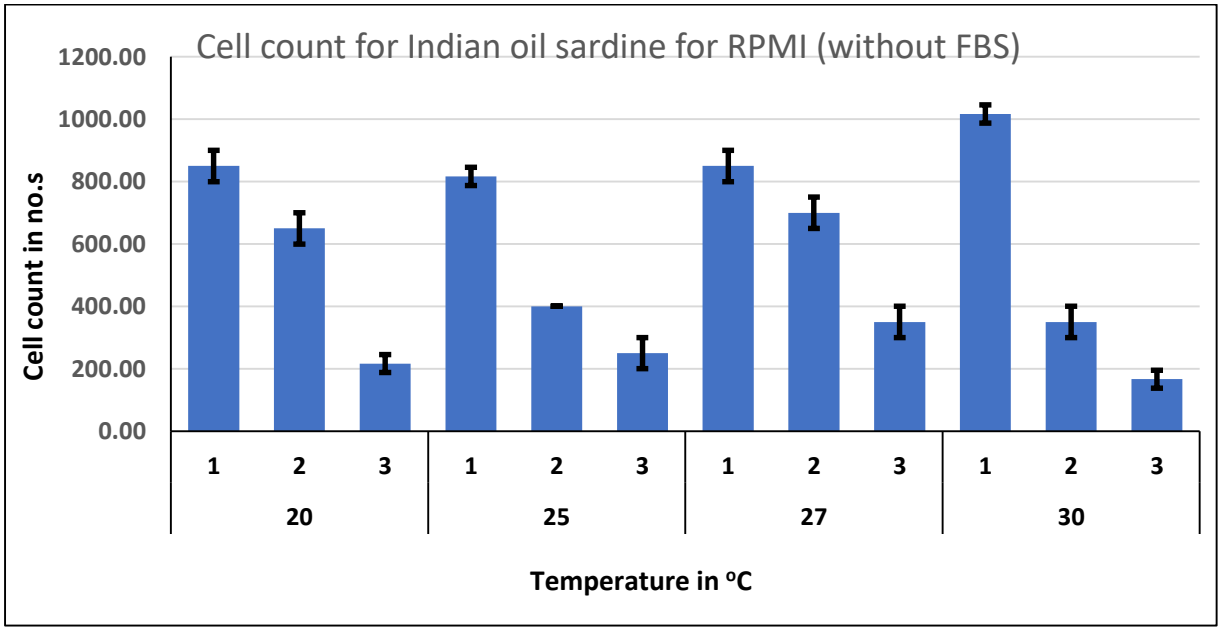


Figure 22: Effect of different temperatures on fish blood cell culture from Indian Oil Sardine established with RPMI medium without FBS. Values shown are Mean ± Standard deviation for n=3. One-way ANOVA between various temperature groups is significant at p<0.0001 (11, F=149).

Table 18: illustrates the influence of different temperatures on fish blood cell culture from Indian Oil Sardine, using DMEM medium without FBS supplementation. The values are represented as Mean ± Standard deviation for n= 3 replicates, with one-way ANOVA indicating significant differences at $p<0.0001$ ($F=125$, $df=11$). On Day 1, varying mean cell counts were observed across different incubating temperatures. At 20°C, the mean cell count was 950.00 ± 50.00, while it decreased to 800.00 ± 50.00 at 25°C. At 27°C, there was a further decrease to 783.33 ± 76.38, followed by a slight increase to 833.33 ± 28.87 at 30°C. By Day 2, the mean cell counts continued to vary. At 20°C, the mean cell count was 433.33 ± 28.87, while it increased to 650.00 ± 50.00 at 25°C. At 27°C, the mean cell count was 450.00 ± 50.00, and it slightly decreased to 400.00 ± 50.00 at 30°C. On Day 3, the mean cell counts displayed further fluctuations. At 20°C, the mean cell count was 0.00, while it increased to 150.00 ± 50.00 at 25°C. At 27°C, the mean cell count was 416.67 ± 28.87, and it decreased to 200.00 ± 50.00 at 30°C.

These findings suggest that DMEM medium without FBS supplementation may have a differential impact on the growth and viability of Indian Oil Sardine blood cells under different temperature conditions (Figure 23).

Table 18: Effect of different temperatures on fish blood cell culture from Indian Oil Sardine established with DMEM medium withot FBS. Values shown are Mean ± Standard deviation for n= 3. One-way ANOVA between various temperature groups is significant at p<0.0001 (11, F=125).

	Incubating temperatures			
	20ºC	**25ºC**	**27ºC**	**30ºC**
Day 1	950.00 ± 50.00	800.00 ±50.00	783.33 ±76.38	833.33 ±28.87
Day 2	433.33 ± 28.87	650.00 ± 50.00	450.00 ± 50.00	400.00 ± 50.00
Day 3	00.00	150.00 ± 50.00	416.67 ± 28.87	200.00 ± 50.00

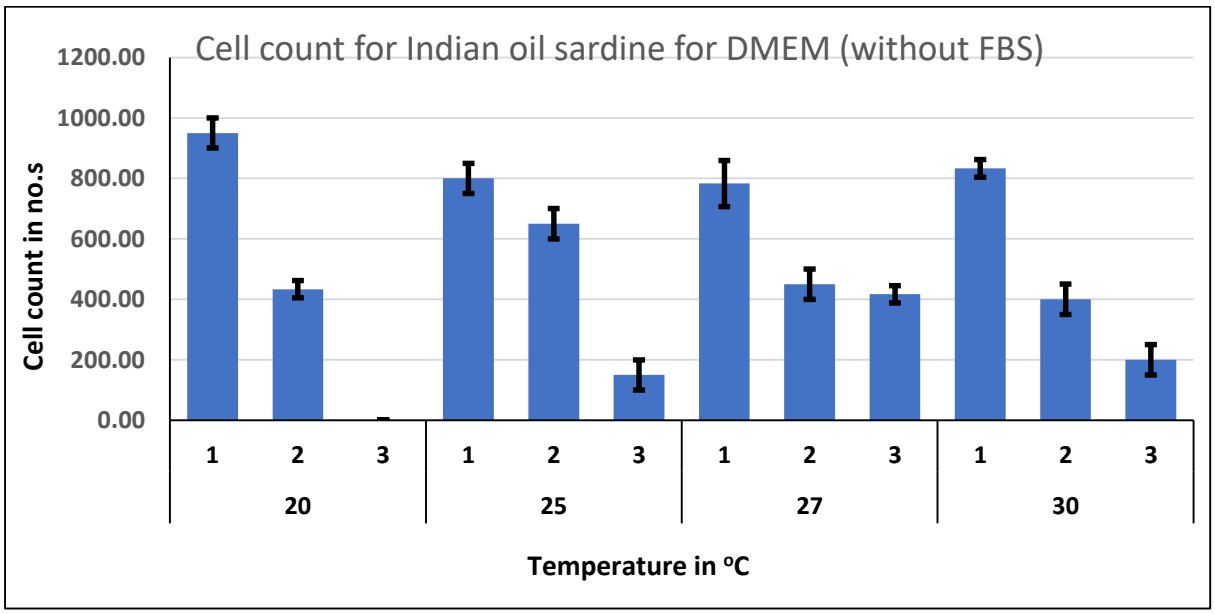


Figure 23: Effect of different temperatures on fish blood cell culture from Indian Oil Sardine established with DMEM medium without FBS. Values shown are Mean ± Standard deviation for n= 3. One-way ANOVA between various temperature groups is significant at p<0.0001 (11, F= 125).

Table 20 presents the effects of different temperatures on fish blood cell culture from Indian Oil Sardine, utilizing Mc'Coys medium without FBS supplementation. The values are represented as Mean ± Standard deviation for n= 3 replicates, with one-way ANOVA indicating significant differences at $p<0.0001$ ($F=119$, $df=11$). On Day 1, varying mean cell counts were observed across different incubating temperatures. At 20°C, the mean cell count was 850.00 ± 50.00, while it decreased to 650.00 ± 50.00 at 25°C. At 27°C, there was an increase to 850.00 ± 50.00, which remained consistent at 30°C. By Day 2, the mean cell counts continued to fluctuate. At 20°C, the mean cell count was 250.00 ± 50.00, while it increased to 333.33 ± 76.38 at 25°C. At 27°C, the mean cell count decreased to 200.00 ± 50.00, and there were no observable cells at 30°C. On Day 3, the mean cell counts further declined. At 20°C, the mean cell count was 133.33 ± 28.87, and there were no observable cells at 25°C, 27°C, and 30°C.

These results suggest that Mc'Coys medium without FBS supplementation may have differential effects on cell viability and growth at different temperatures (Figure 24).

Table 19: Effect of different temperatures on fish blood cell culture from Indian Oil Sardine established with Mc'coys medium without FBS. Values shown are Mean ± Standard deviation for n= 3. One-way ANOVA between various temperatures groups is significant at p<0.0001 (11, F=119)

	Incubating temperatures			
	20ºC	**25ºC**	**27ºC**	**30ºC**
Day 1	850.00 ± 50.00	650.00 ±50.00	850.00 ±50.00	850.00 ±50.00
Day 2	250.00 ± 50.00	333.33 ± 76.38	200.00 ± 50.00	00.00
Day 3	133.33 ± 28.87	00.00	00.00	00.00

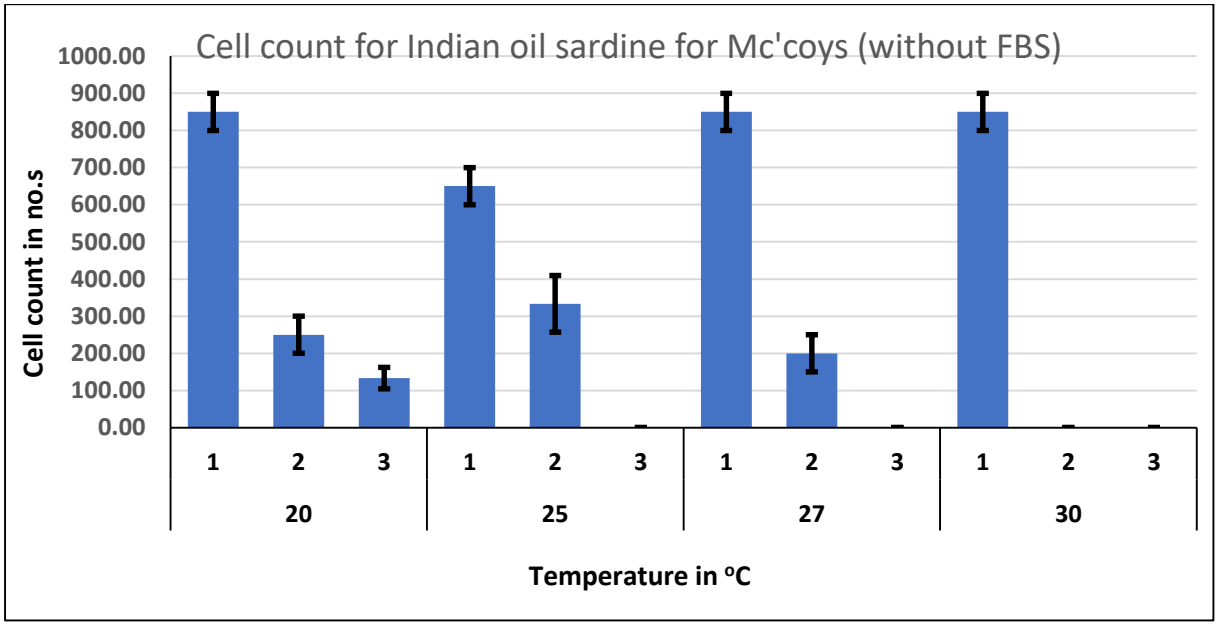


Figure 24: Effect of different temperatures on fish blood cell culture from Indian Oil Sardine established with Mc'coys medium without FBS. Values shown are Mean ± Standard deviation for n= 3. One-way ANOVA between various temperature groups is significant at p<0.0001 (11, F=119)

The karyotype analysis of Indian Mackerel (*Rastrelliger kanagurta*) revealed a diploid chromosome number of 46 (Figure 26). Metaphase spreads obtained from blood cell cultures established with RPMI, DMEM, and McCoy's medium were subjected to Giemsa staining and microscopic examination to visualize chromosome morphology and organization. Under a light microscope, well-spread metaphase chromosomes were observed, displaying distinct banding patterns characteristic of Indian Mackerel chromosomes. The karyotype exhibited a range of chromosome sizes, with clear distinctions between individual chromosomes. Chromosome counting and arrangement based on size facilitated the construction of the karyotype, which revealed 48 chromosomes arranged in homologous pairs. The karyotype analysis provided valuable insights into the chromosomal features of Indian Mackerel, laying the foundation for further cytogenetic studies and contributing to our understanding of the genetic architecture of this economically important fish species. The diploid chromosome number of 48 observed in Indian Mackerel aligns with previous cytogenetic studies and serves as a reference for future research on chromosomal evolution, population genetics, and conservation efforts in this species.

Figure 25- Chromosome spread (Indian mackerel): a) Chromosome spread and b) Counted chromosome spread

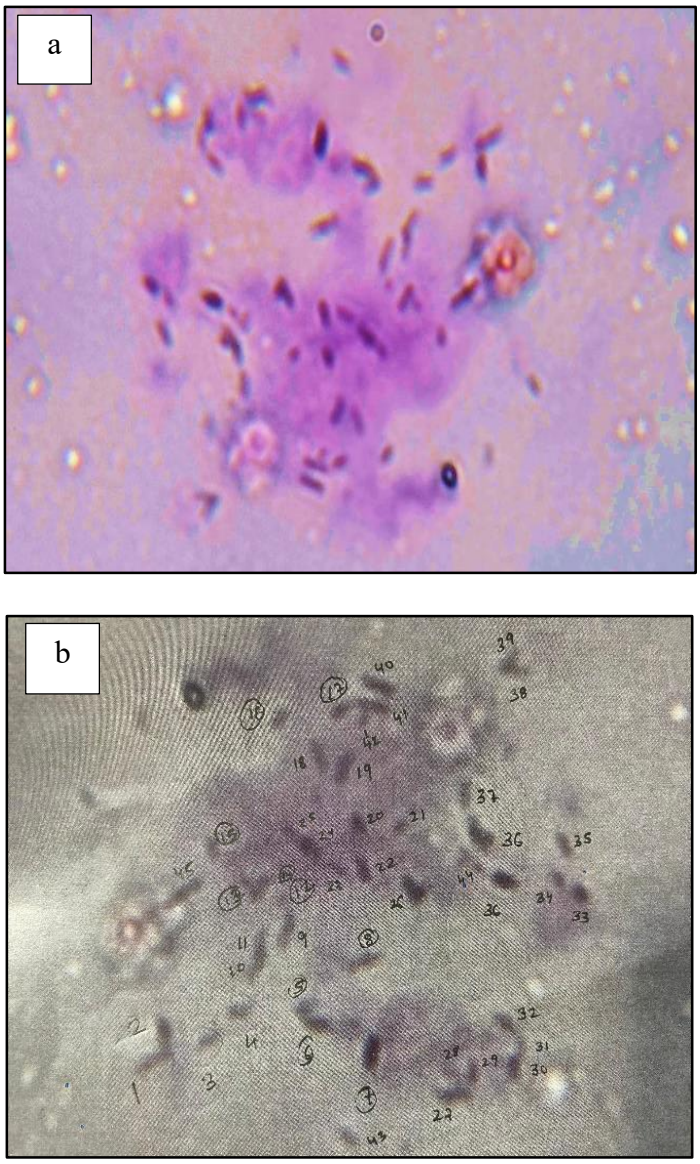
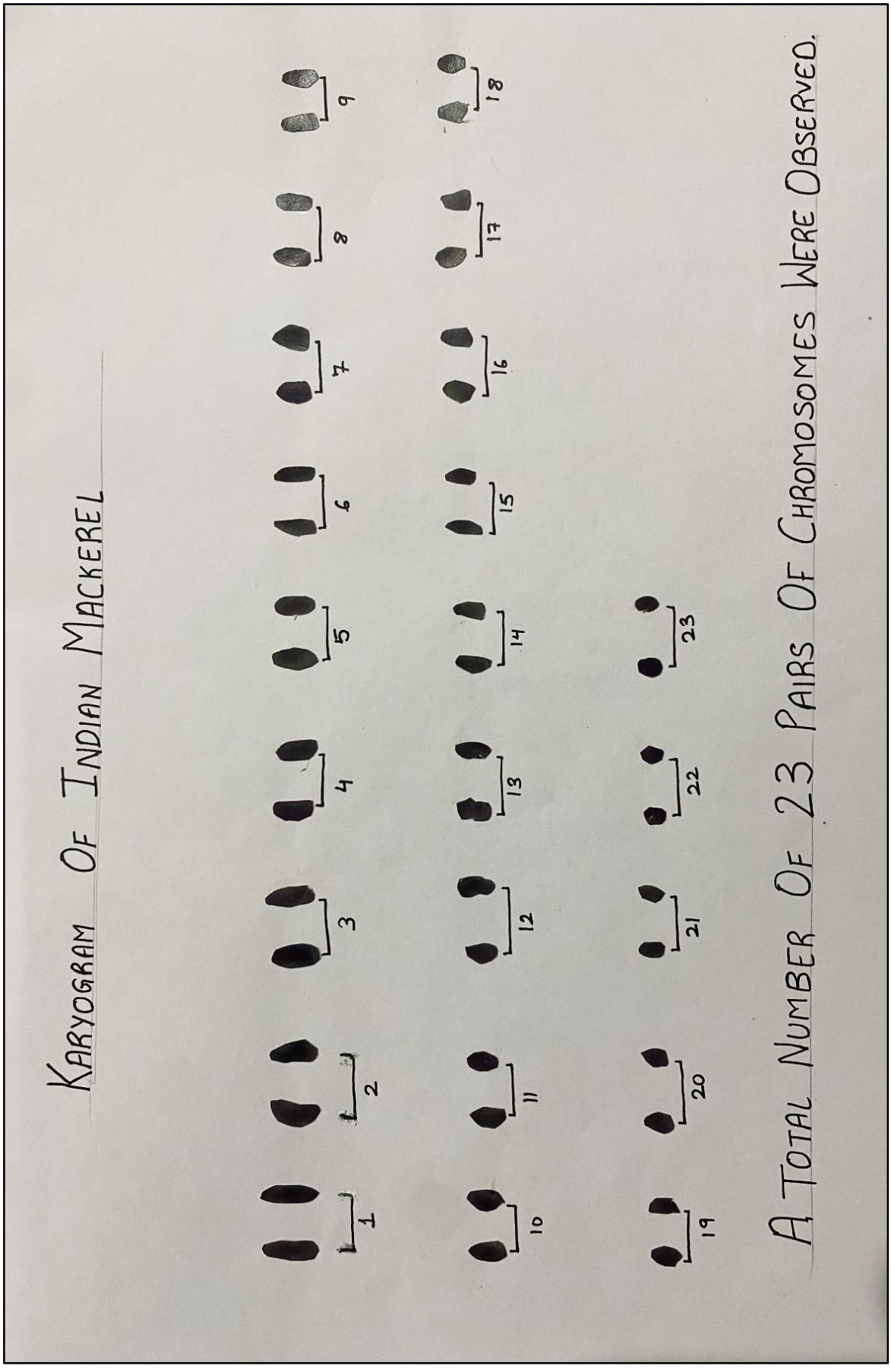


Figure 26- Karyogram of the Indian mackerel



The karyotype analysis of Indian Oil Sardine (Sardinella longiceps) unveiled a diploid chromosome count of 48 (Plate 28). Through Giemsa staining and microscopic examination of metaphase spreads derived from blood cell cultures cultivated with RPMI, DMEM, and McCoy's medium, we discerned the chromosomal landscape of this species. Upon microscopic observation, well-distributed metaphase chromosomes exhibited unique banding patterns indicative of distinct genetic segments. Each chromosome displayed varying sizes, enabling clear identification and arrangement into homologous pairs. The karyotype, comprising 48 chromosomes, elucidated the chromosomal architecture of Indian Oil Sardine. This analysis furnishes pivotal insights into the chromosomal makeup of Indian Oil Sardine, providing a cornerstone for further cytogenetic investigations. The consistent diploid count of 48 chromosomes aligns with prior research and establishes a foundational framework for exploring chromosomal evolution, population genetics, and conservation strategies in this significant fish species.

Figure 27- Chromosome spread (Indian oil sardine):

a) Chromosome spread and b) Counted chromosome spread

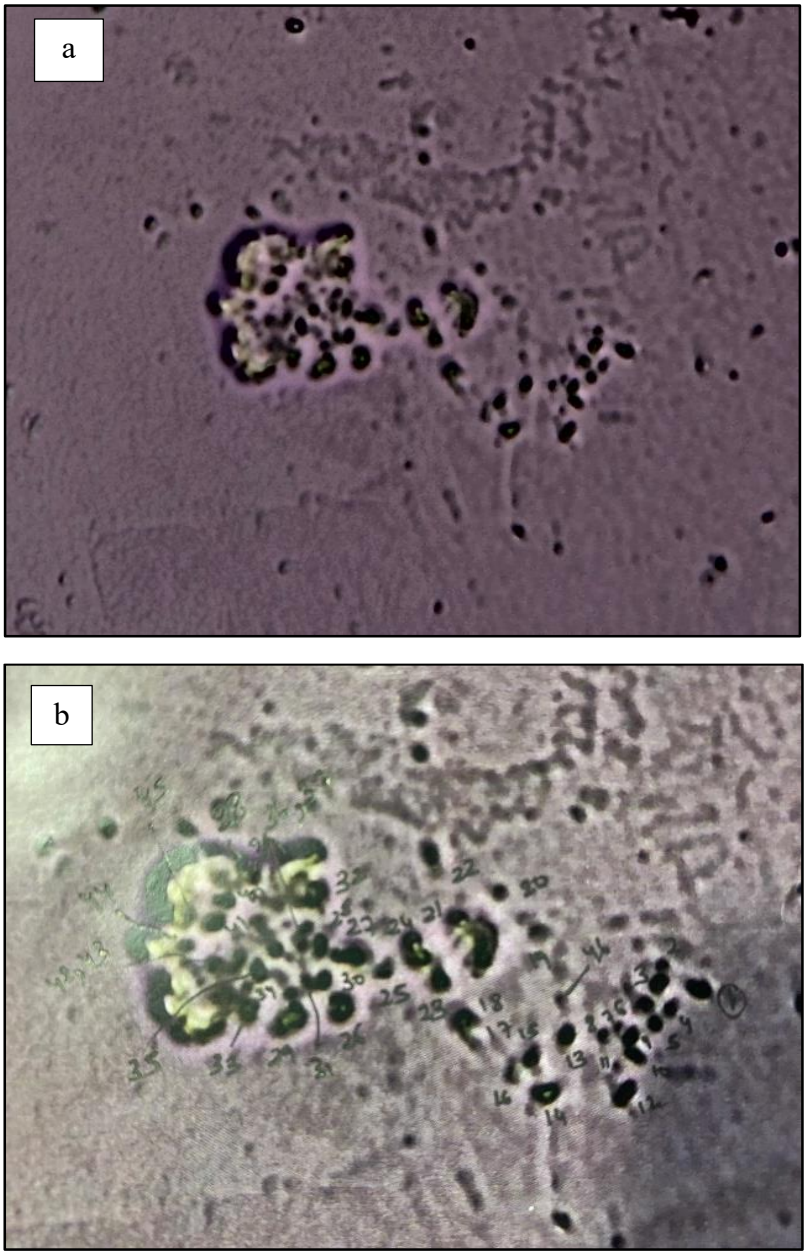
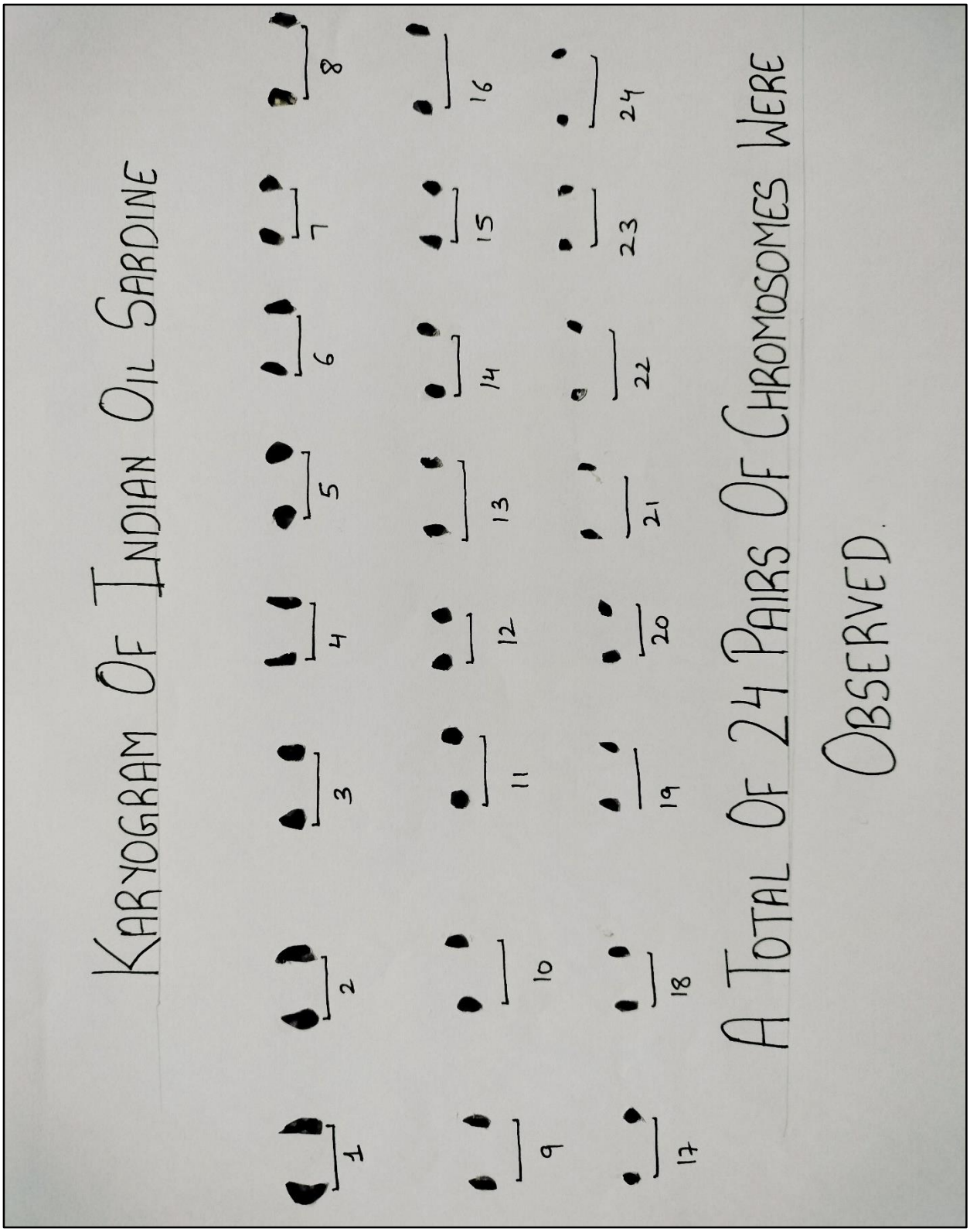


Figure 28- Karyogram of the Indian oil sardine



For the Flathead Grey Mullet (Mugil cephalus), our karyotype analysis revealed a diploid chromosome count of 48 (Plate 30). Through meticulous examination of metaphase spreads derived from blood cell cultures established with RPMI, DMEM, and McCoy's medium, we gained intricate insights into the chromosomal composition of this species. Under microscopic scrutiny, the metaphase chromosomes exhibited distinct banding patterns indicative of their unique genetic characteristics. Each chromosome displayed varying sizes, facilitating their identification and arrangement into homologous pairs. The karyotype, comprising 46 chromosomes, offered a comprehensive view of the chromosomal architecture of the Flathead Grey Mullet. This analysis serves as a vital foundation for further investigations into the genetic makeup and chromosomal evolution of the Flathead Grey Mullet. The consistent diploid count of 46 chromosomes provides essential baseline data for future studies focusing on population genetics, phylogenetic relationships, and conservation efforts aimed at preserving this ecologically significant fish specie

Figure 29- Chromosome spread (Flathead grey mullet):

a) Chromosome spread and b) Counted chromosome spread

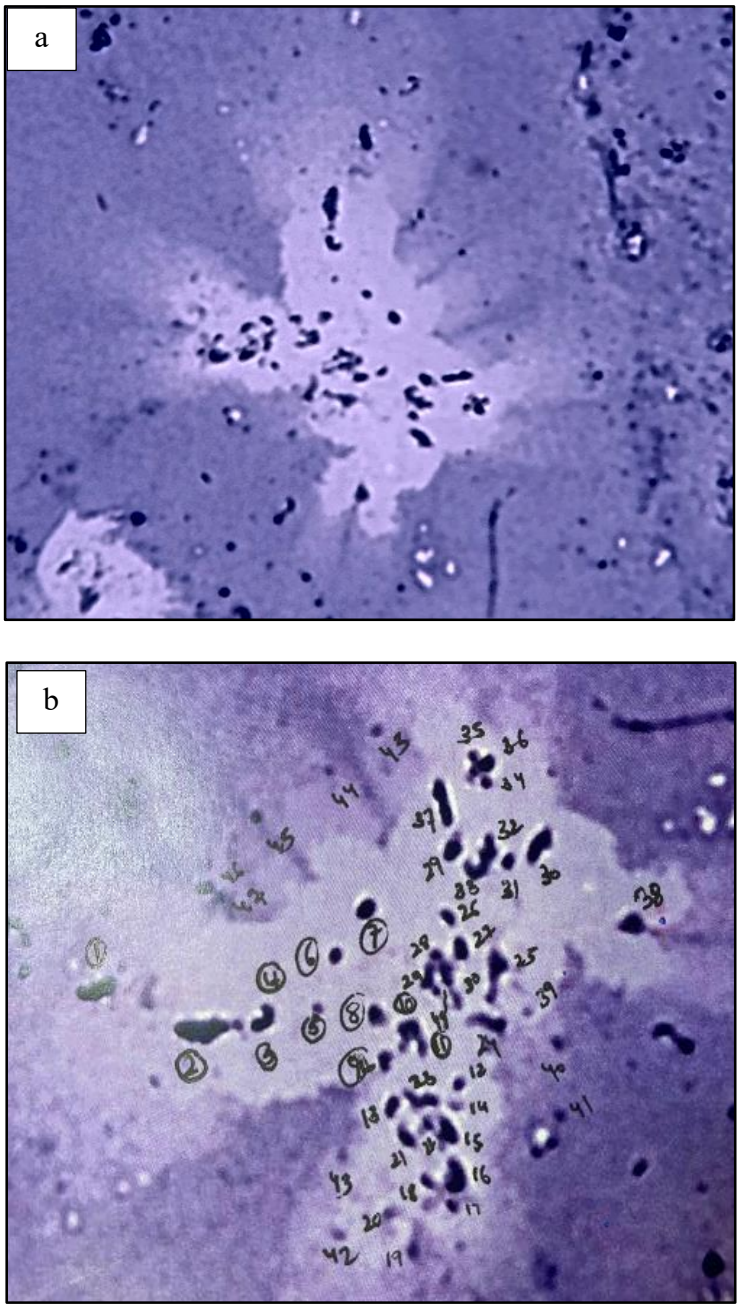
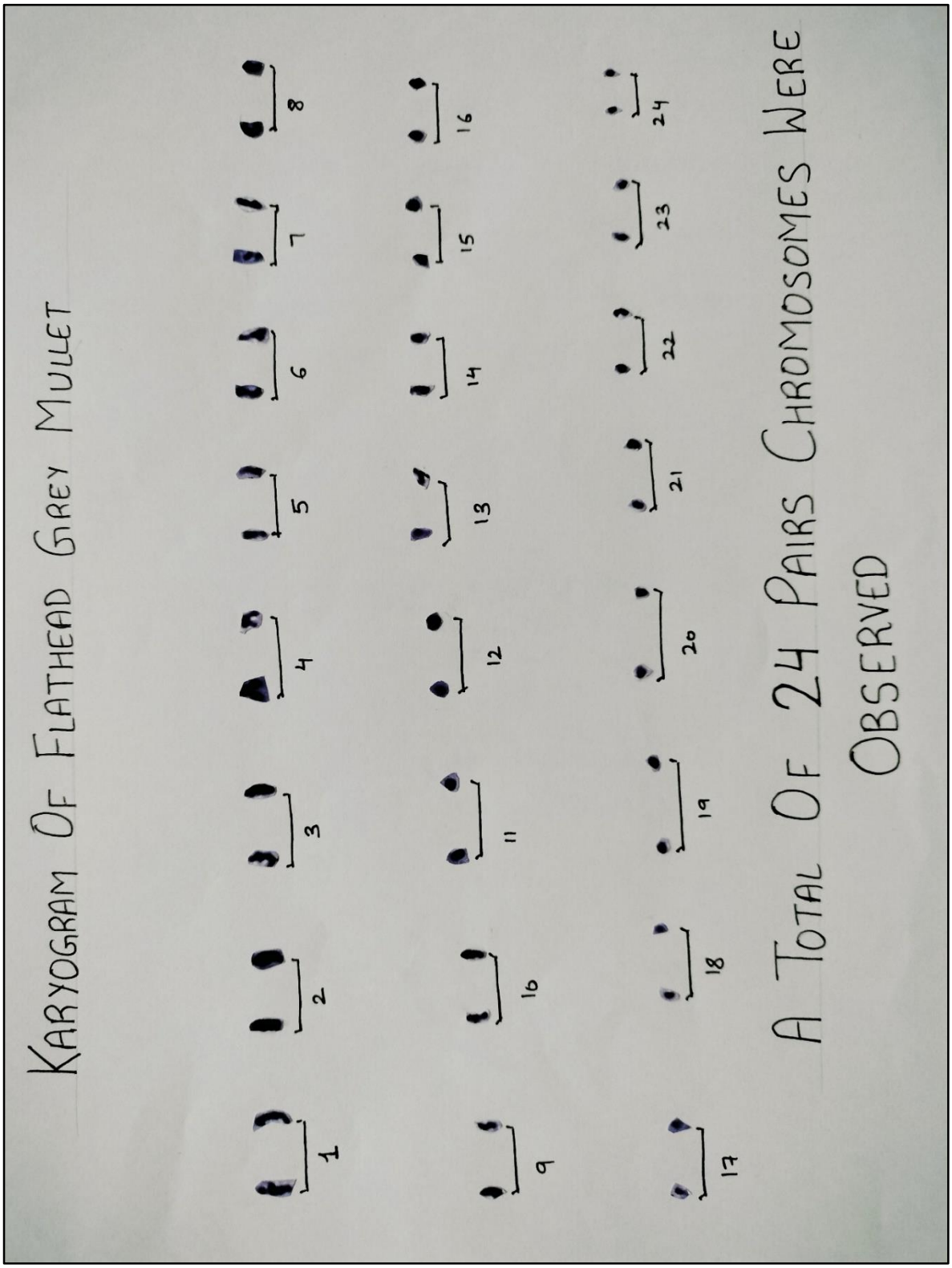


Figure 30- Karyogram of the Flathead grey mullet



Fish cytogenetics, a multidisciplinary field at the intersection of genetics, ecology, and evolutionary biology, investigates the chromosomal characteristics of fish species to unravel genetic diversity, evolutionary relationships, and adaptation mechanisms. Studies such as Ryu *et al*. (2017) emphasize the cautious use of antibiotics in cell culture due to their potential to induce changes in gene expression and regulation, underscoring the importance of methodological precision. Lopresto *et al*. (1995) developed specialized in vitro culture systems for fish leucocytes, refining techniques crucial for cytogenetic research in fish. Additionally, the compilation of cytogenetic methodologies by Ozouf-Costaz *et al.* (2015) provides researchers with invaluable tools for studying fish chromosomes across diverse taxa.

Insights into karyotyping procedures, as discussed by Fujiwara *et al*. (2001) and Veerabhadrappa *et al*. (2016), have enhanced the efficiency and reliability of chromosome preparation and diagnosis of chromosomal disorders in fish populations. Moreover, the comprehensive review by Rossi (2021) illuminates the current state and future directions of fish cytogenetics, highlighting its significance in evolutionary biology, aquaculture, and conservation genetics. These studies, alongside others exploring fish karyotypes and chromosomal diversity, contribute to our understanding of the genetic landscape of aquatic ecosystems, providing essential knowledge for fisheries management and biodiversity conservation efforts.

Furthermore, the application of advanced cytogenetic techniques, such as fluorescence in situ hybridization (FISH), has enabled the precise mapping of genes and repetitive DNA sequences on fish chromosomes, facilitating comparative genomics and evolutionary studies (Sinclair, 2002; Scapigliati *et al*., 2018). The characterization of fish karyotypes, as exemplified by Arai (2011) and Nagpure *et al*. (2016), offers valuable taxonomic information and insights into chromosomal evolution within fish families and genera. In addition to fundamental research, fish cytogenetics plays a vital role in applied fields, including aquaculture and fisheries management. Understanding the chromosomal basis of traits related to growth, reproduction, and disease resistance can inform selective breeding programs aimed at improving aquaculture species' productivity and resilience

to environmental stressors (Mascarenhas & Desai, 2023). Moreover, studies on the karyotypes of economically important fish species, such as those conducted by Heckel (1843) and Miyaki *et al*. (1995), contribute to the sustainable management of wild fish populations and the development of conservation strategies.

The study investigated three distinct fish species: Indian Mackerel (*Rastrelliger kanagurta*), Indian Oil Sardine (*Sardinella longiceps*), and Grey Mullet (*Mugil cephalus*), each representing diverse ecological niches and economic importance within the marine ecosystem (Mascarenhas & Desai, 2023). Indian Mackerel, a pelagic species thriving in warm shallow waters, embodies the rich biodiversity of coastal regions along the Indian and western Pacific oceans (Nagpure et al., 2016). Similarly, the Indian Oil Sardine, a key contributor to marine fish production in India, underscores the socio-economic significance of pelagic fisheries (Bazaz et al., 2022). On the other hand, the Grey Mullet's catadromous behavior, characterized by migration from freshwater to coastal estuaries, highlights the species' adaptation to diverse aquatic habitats (Sahoo et al., 2007).

Indian Mackerel, known scientifically as *Rastrelliger kanagurta*, stands as a quintessential example of a pelagic species that thrives in warm shallow waters (Mascarenhas & Desai, 2023). Its presence is pervasive along the coastal regions lining the Indian and western Pacific oceans, where it plays a crucial role in the marine food web (Heckel, 1843). Renowned for its swift swimming abilities and agile hunting techniques, the Indian Mackerel occupies a prominent position in the ecosystem, serving as both predator and prey (Bazaz *et al*., 2022). Its adaptation to the dynamic and often challenging conditions of coastal waters underscores its resilience and evolutionary prowess, making it a focal point of ecological studies and conservation efforts (Nagpure *et al*., 2016).

Similarly, the Indian Oil Sardine (*Sardinella longiceps*) emerges as a cornerstone of marine fish production in India, exemplifying the socio-economic significance of pelagic fisheries (Bazaz *et al*., 2022). With its distinct silver-blue hue and slender, streamlined body, the Indian Oil Sardine

navigates the expansive waters of the Indian Ocean with remarkable agility (Mascarenhas & Desai, 2023). Its schooling behavior and voracious appetite make it a sought-after catch among commercial fishermen, supporting local economies and providing a vital source of protein for coastal communities (Nagpure et al., 2016). The intricate interplay between environmental factors, population dynamics, and fishing pressure underscores the complex relationship between humans and marine resources, prompting researchers to explore sustainable management practices and conservation strategies (Bazaz et al., 2022).

In contrast, the Grey Mullet (*Mugil cephalus*) offers a glimpse into the fascinating world of catadromous fish species, characterized by their unique migratory patterns from freshwater habitats to coastal estuaries (Sahoo et al., 2007). With its elongated body, silvery scales, and distinctive cephalic lobes, the Grey Mullet epitomizes adaptability in the face of changing environmental conditions (Mascarenhas & Desai, 2023). Its ability to traverse diverse aquatic environments, from freshwater rivers to brackish estuaries, highlights its evolutionary versatility and resilience (Sahoo *et al*., 2007). As a keystone species in estuarine ecosystems, the Grey Mullet plays a pivotal role in nutrient cycling, sediment stabilization, and biodiversity maintenance, shaping the ecological dynamics of coastal wetlands and supporting a myriad of plant and animal species (Bazaz *et al*., 2022).

The successful extraction of fresh blood samples from the three distinct fish species, namely Indian Mackerel (*Rastrelliger kanagurta*), Indian Oil Sardine (*Sardinella longiceps*), and Grey Mullet (*Mugil cephalus*), marked a significant advancement in the study's methodology. This crucial step laid the foundation for the establishment of blood cell cultures, a fundamental aspect of cytogenetic research aimed at understanding the chromosomal characteristics and genetic diversity of these species (Mascarenhas & Desai, 2023).

To establish the blood cell cultures, three different growth media were employed: RPMI, DMEM, and McCoy’s medium. These media were selected based on their suitability for reviving and

maintaining various peripheral blood cell subtypes, including but not limited to T cells, B cells, and monocytes (Bayot *et al*., 2020). The utilization of multiple growth media allowed for comprehensive exploration and optimization of culture conditions to maximize cell viability and growth.

Observation under the microscope revealed robust cell growth and minimal contamination within the established cultures, indicating the suitability of the selected culture conditions. The cells exhibited healthy morphology and proliferation, indicative of their ability to thrive in the laboratory setting (Fujiwara *et al*., 2001). This observation underscored the importance of stringent aseptic techniques during sample collection and culture initiation, ensuring the integrity of the experimental results.

One of the most significant findings of the study was the observation of metaphase within 24 hours of incubation. Metaphase is a critical stage of the cell cycle during which chromosomes align in the center of the cell, preparing for their subsequent segregation into daughter cells during mitosis (Heng *et al*., 2013). The prompt arrival of cells at the metaphase stage indicated the rapid proliferation and synchronization of the cultured cells, essential for subsequent karyotyping procedures.

The ability to induce metaphase within a relatively short incubation period of 24 hours holds immense practical significance for cytogenetic studies. Traditionally, achieving metaphase synchronization in cell cultures has been a time-consuming process, often requiring prolonged incubation periods and specialized protocols (Veerabhadrappa *et al*., 2016). However, the optimized culture conditions employed in this study facilitated the rapid progression of cells through the cell cycle, expediting the metaphase arrest and subsequent karyotyping procedures.

The timely induction of metaphase not only streamlines the experimental workflow but also minimizes the risk of cell cycle-related artifacts and abnormalities, ensuring the accuracy and reliability of the karyotypic analysis (Galetti *et al*., 2000). Moreover, the efficiency of metaphase

induction reflects the robustness of the established culture system and the compatibility of the selected growth media with the biological characteristics of the cultured cells.

Metaphase arrest, a crucial step in cytogenetic analysis, plays a pivotal role in synchronizing cell division and enhancing chromosome visualization. This process is achieved through the addition of colchicine, a well-known alkaloid drug with potent effects on cell division dynamics (Rossi, 2021). Colchicine's mechanism of action revolves around its ability to disrupt spindle fiber formation, thereby preventing the proper alignment and segregation of chromosomes during mitosis (Ryu *et al*., 2017). By inducing metaphase arrest, colchicine effectively halts cell division at a stage where chromosomes are condensed and aligned at the cell's equatorial plate, facilitating the subsequent analysis of chromosome morphology and number (Heng *et al*., 2013).

The addition of colchicine to the cell culture medium marks a critical phase in the experimental workflow, as it serves to synchronize the cell population at a specific stage of the cell cycle. This synchronization is essential for obtaining a homogeneous population of cells in metaphase, thereby minimizing variability in chromosome morphology and simplifying the karyotyping process (Sember *et al*., 2015). Moreover, the precise timing of colchicine treatment is crucial to ensure optimal metaphase arrest without compromising cell viability or inducing cytotoxic effects (Fujiwara *et al*., 2001).

Following metaphase arrest, the next steps in the cytogenetic protocol involve the preparation of intact cell pellets for chromosome analysis. This process typically entails centrifugation and fixation procedures to concentrate the cells and preserve their morphology for subsequent staining and visualization (Ozouf-Costaz *et al*., 2015). Hypotonic treatment, involving the use of potassium chloride solution, is commonly employed to induce cell swelling and facilitate the separation of chromosomes from the cytoplasm (Arai, 2011). This step is crucial for obtaining well-dispersed chromosomes with minimal clumping, ensuring optimal staining and microscopic analysis (Miyaki *et al*., 1995).

Following hypotonic treatment, the cells are subjected to centrifugation, a process that involves spinning the cell suspension at high speeds to separate the cellular components based on their density (Nagpure *et al*., 2016). Centrifugation serves to concentrate the chromosomes into a compact pellet at the bottom of the centrifuge tube, allowing for the removal of excess cytoplasmic debris and supernatant (Scapigliati *et al*., 2018). This step is essential for maximizing the yield of intact chromosomes while minimizing background noise and artifacts during subsequent staining procedures (Tanomtong *et al*., 2014).

Once the cell pellet is obtained, fixation is carried out to preserve the cellular morphology and prevent degradation of the chromosomes. Carnoy's solution, a mixture of ethanol and acetic acid, is commonly used as a fixative due to its ability to rapidly penetrate the cells and denature proteins, ensuring the preservation of cellular structures (Bhatnagar *et al*., 2014). Fixation serves to immobilize the chromosomes in their condensed state, making them amenable to staining and visualization under the microscope (Sing et al., 2013).

The adoption of advanced cytogenetic techniques, such as the splash technique, represents a significant advancement in optimizing chromosome spreading and enhancing the accuracy of karyotype preparation. The splash technique, developed as an improvement over conventional methods, involves the mechanical disruption of cells on a glass slide, resulting in the uniform spreading of chromosomes across the slide surface (Ulsh et al., 2000b). This innovative approach ensures the generation of well-spread metaphases, characterized by minimal overlap and optimal chromosome dispersion, thus facilitating precise chromosome analysis.

The implementation of the splash technique begins with the preparation of a cell suspension, obtained from the previously fixed and centrifuged cell pellet. A small aliquot of the cell suspension is then deposited onto a clean glass slide, followed by gentle agitation or "splashing" to disperse the cells evenly across the slide surface. This mechanical disruption serves to break

apart cell aggregates and encourage the uniform distribution of chromosomes, thereby maximizing the chances of obtaining well-spread metaphases (Nagpure et al., 2016).

Staining with Giemsa dye represents the next critical step in the cytogenetic workflow, as it enhances chromosome visualization and enables the identification of distinct staining patterns characteristic of individual chromosomes. Giemsa dye, a DNA-intercalating stain, binds preferentially to the adenine-thymine-rich regions of chromosomal DNA, resulting in the differential staining of chromatin regions with varying base pair compositions (Ulsh et al., 2000a).

The process of chromosome counting and arrangement based on size represents the culmination of the cytogenetic analysis, leading to the construction of karyotypes for the examined fish species. Karyotyping involves the systematic classification and arrangement of chromosomes according to their size, centromere position, and banding patterns, providing valuable insights into chromosomal structure and organization (Bazaz *et al*., 2022). By visually inspecting the stained metaphase spreads under a microscope, cytogeneticists can enumerate the total number of chromosomes present in each karyotype and identify any structural abnormalities or rearrangements.

The construction of karyotypes offers a wealth of information regarding the genetic composition and evolutionary history of the examined fish species. By comparing karyotypes across different populations or species, researchers can elucidate patterns of chromosomal variation and divergence, shedding light on speciation events, population genetics, and phylogenetic relationships (Heckel, 1843). Additionally, karyotypic analysis can uncover potential chromosomal markers associated with specific traits or phenotypes, providing valuable insights for breeding programs, conservation efforts, and evolutionary studies.

The successful establishment of blood cell cultures from Indian Mackerel (*Rastrelliger kanagurta*), Indian Oil Sardine (*Sardinella longiceps*), and Grey Mullet (*Mugil cephalus*) represents a significant breakthrough in cytogenetic research. This achievement opens up exciting

new avenues for investigating the chromosomal characteristics and genetic diversity of these ecologically and economically important fish species. The rapid attainment of metaphase within 24 hours of incubation under optimized culture conditions highlights the efficiency and reliability of the established protocols, enhancing their practical utility for karyotypic analysis and contributing to our understanding of marine biodiversity.

The ability to culture blood cells from these fish species provides researchers with a valuable tool for studying their chromosomal makeup and genetic variability. By observing metaphase within a relatively short timeframe, researchers can efficiently analyze chromosome structure and organization, leading to insights into the evolutionary history and population dynamics of these species. This rapid attainment of metaphase is particularly advantageous for karyotypic analysis, as it allows for the accurate visualization and characterization of chromosomes, paving the way for detailed genetic studies.

Furthermore, the establishment of blood cell cultures from Indian Mackerel, Indian Oil Sardine, and Grey Mullet holds significant implications for marine biodiversity research and conservation efforts. These species play key roles in marine ecosystems, contributing to ecosystem stability and supporting livelihoods in coastal communities. Understanding their genetic structure and population dynamics is essential for effective conservation management and sustainable fisheries practices.

By elucidating the chromosomal characteristics and genetic diversity of these fish species, researchers can gain valuable insights into their evolutionary relationships, population structure, and adaptive responses to environmental changes. This knowledge can inform conservation strategies aimed at preserving genetic diversity, maintaining healthy fish populations, and safeguarding the long-term sustainability of marine ecosystems.

Moreover, the establishment of blood cell cultures from Indian Mackerel, Indian Oil Sardine, and Grey Mullet has broader implications for aquaculture and fisheries management. By studying the

genetic variability within and among populations of these species, researchers can identify genetic markers associated with desirable traits such as disease resistance, growth rate, and reproductive success. This information can be used to develop selective breeding programs aimed at enhancing the productivity and resilience of aquaculture stocks, ultimately benefiting both the aquaculture industry and wild fish populations.

Based on the study conducted, the establishment of blood cell cultures from Indian Mackerel, Indian Oil Sardine, and Grey Mullet presents a viable solution to the problem of limited access to chromosomal data and genetic information in these ecologically and economically important fish species. By successfully culturing blood cells and observing metaphase within 24 hours of incubation, the study demonstrates the feasibility and efficiency of the optimized culture conditions for karyotypic analysis. This solution addresses the challenge of accessing chromosomal data, which is crucial for understanding these fish species' genetic diversity, population dynamics, and evolutionary relationships. With the ability to culture blood cells and analyze chromosomes, researchers can now investigate the chromosomal characteristics, genetic variability, and adaptive responses to environmental changes in Indian Mackerel, Indian Oil Sardine, and Grey Mullet more comprehensively.

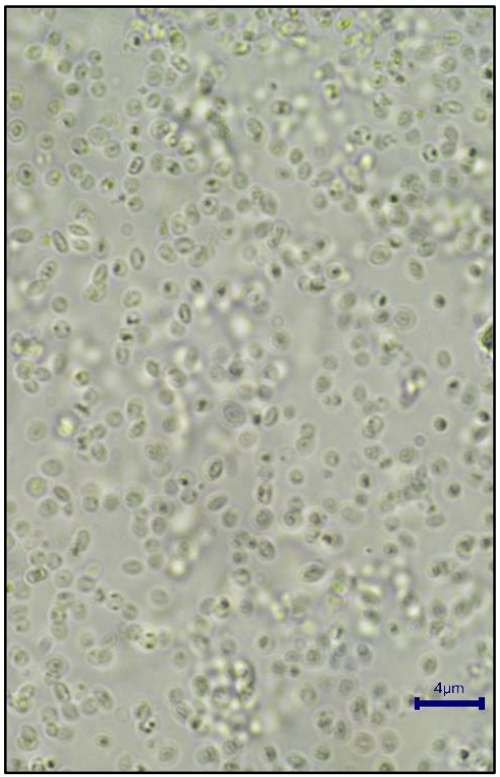
Furthermore, the establishment of blood cell cultures opens up new avenues for studying the genetic basis of important traits such as disease resistance, growth rate, and reproductive success in these fish species. By identifying genetic markers associated with desirable traits, researchers can develop selective breeding programs aimed at enhancing the productivity and resilience of aquaculture stocks, contributing to the sustainability of fisheries and aquaculture industries.

In addition to its implications for aquaculture and fisheries management, the study's findings have broader implications for marine biodiversity conservation. By deepening our understanding of the genetic diversity and population structure of Indian Mackerel, Indian Oil Sardine, and Grey Mullet, the study informs conservation strategies aimed at preserving genetic diversity,

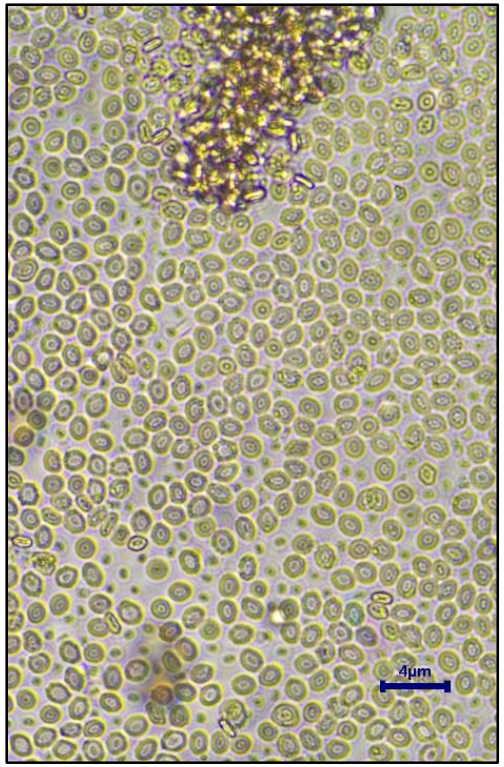
maintaining healthy fish populations, and safeguarding the long-term sustainability of marine ecosystems.

In the present study, the establishment of blood cell cultures and the rapid attainment of metaphase within 24 hours of incubation represent significant advancements in cytogenetic research, offering a promising solution to the problem of limited access to chromosomal data in Indian Mackerel, Indian Oil Sardine, and Grey Mullet. Moving forward, these findings provide a solid foundation for further research aimed at unraveling the genetic mysteries of these ecologically important fish species and guiding conservation and management efforts in coastal ecosystems.

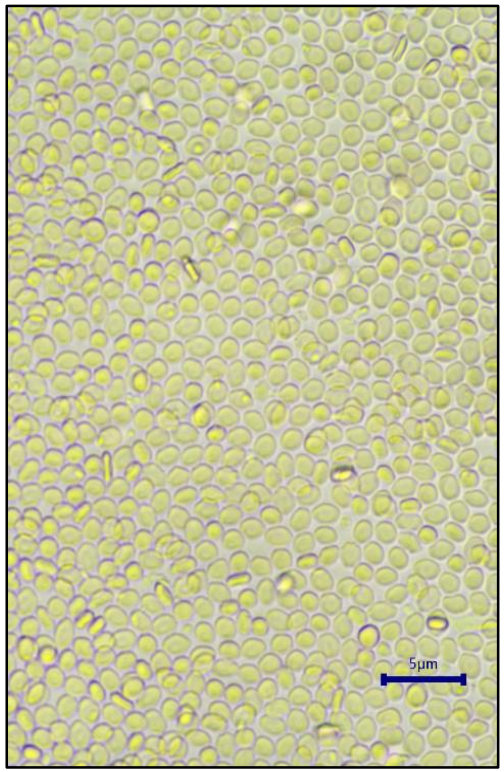
Figure 31- Indian mackerel cells under 40X magnification (with RPMI)



Day 3

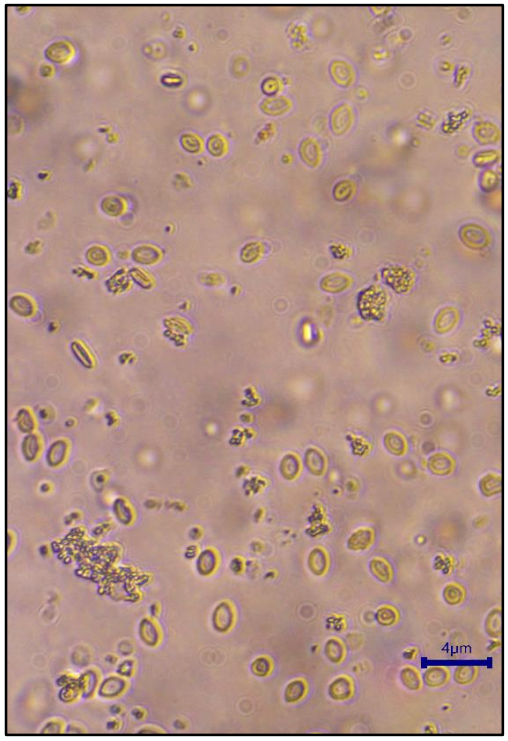


Day 2

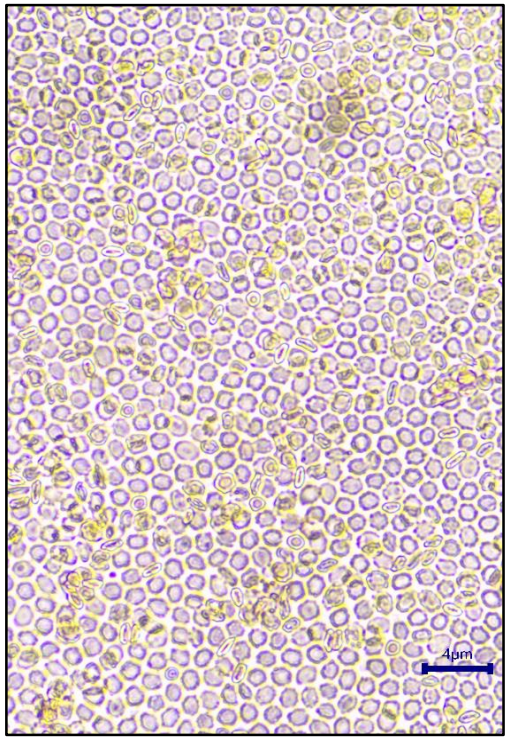


Day 1

Figure 32-Indian Mackerel cells under 40X magnification (with DMEM)



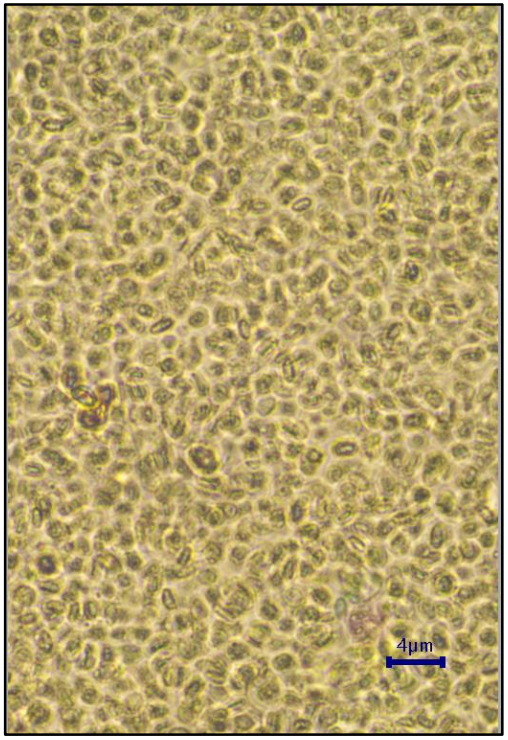
Day 1



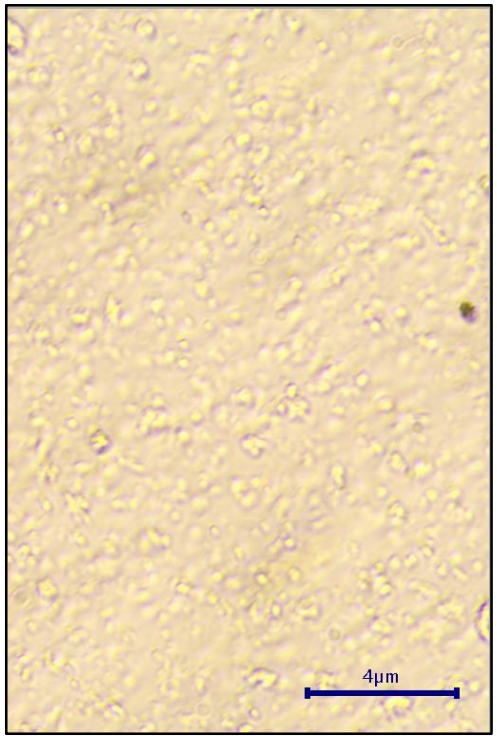
Day 1

Day 1

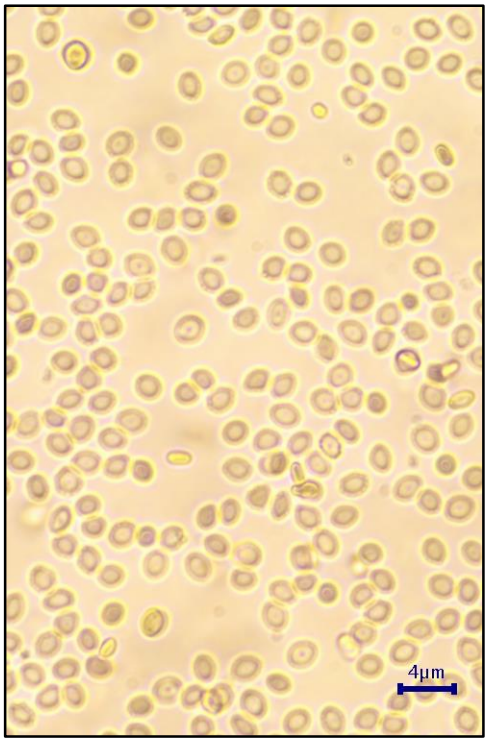
Figure 33-Indian Mackerel cells under 40X magnification (with Mc'coys media)



Day 3

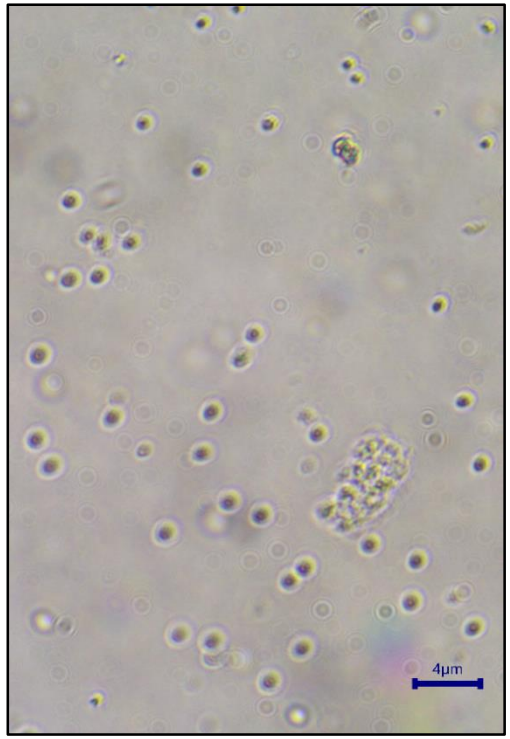


Day 2

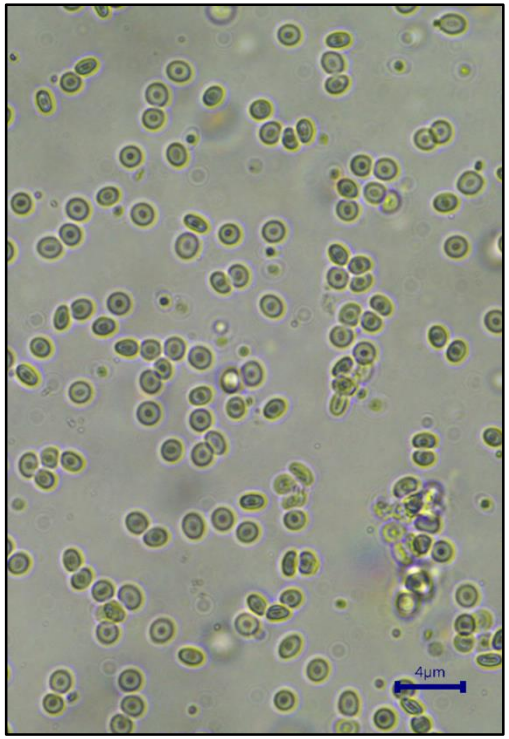


Day 1

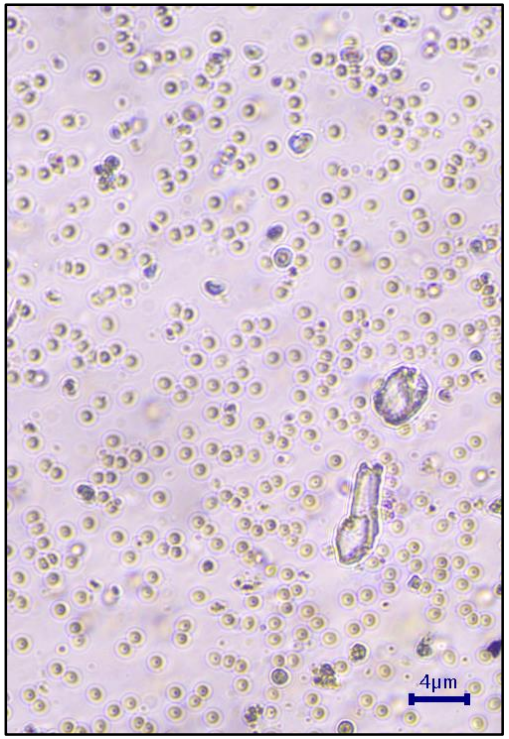
Figure 34-Indian Oil Sardine cells under 40X magnification (with RPMI)



Day 3

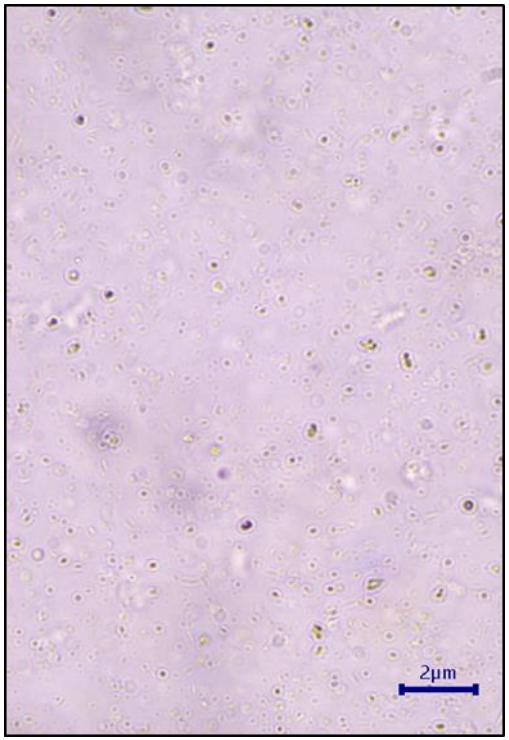


Day 2

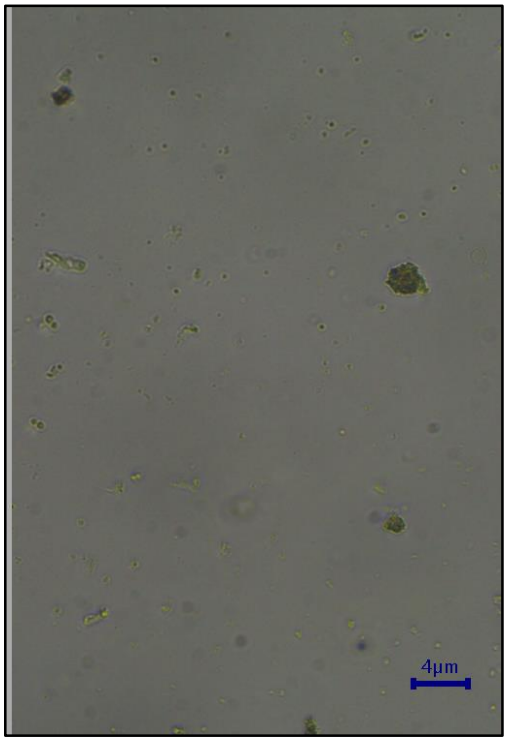


Day 1

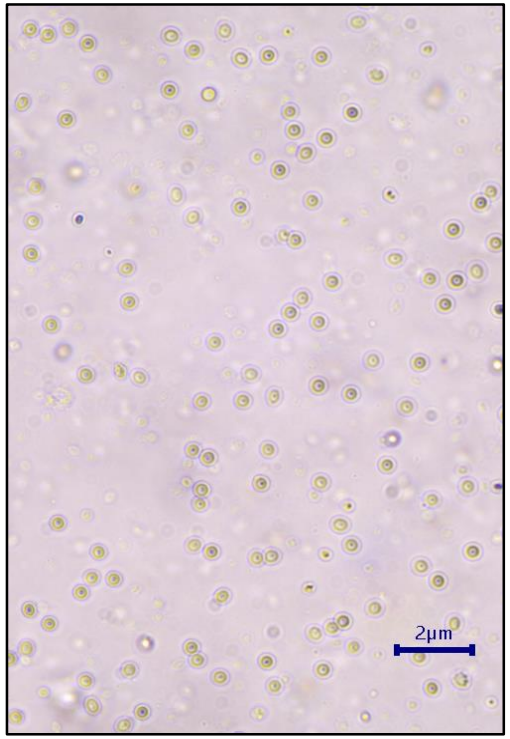
Figure 35-Indian Oil Sardine cells under 40X magnification (with DMEM)



Day 3

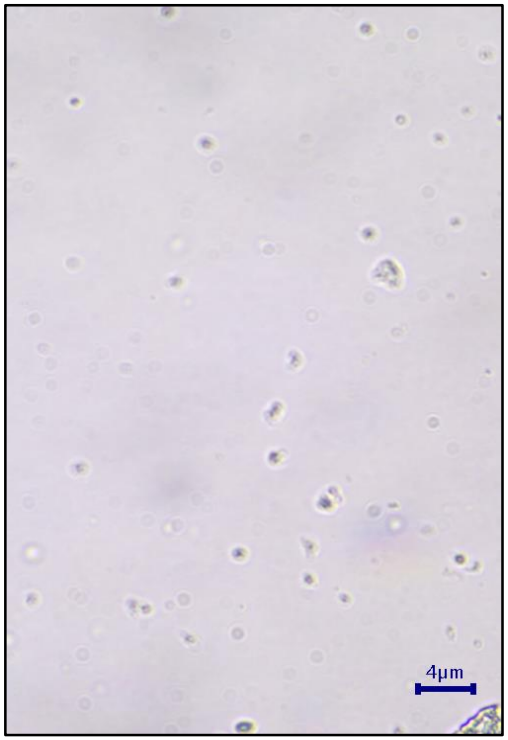


Day 2

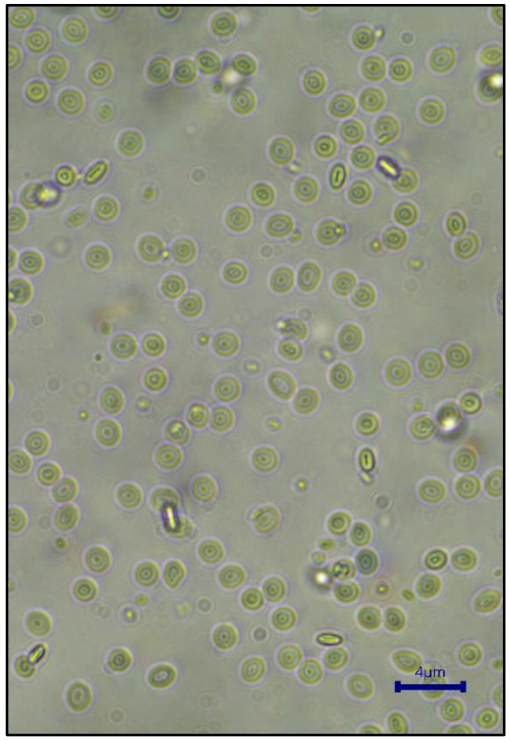


Day 1

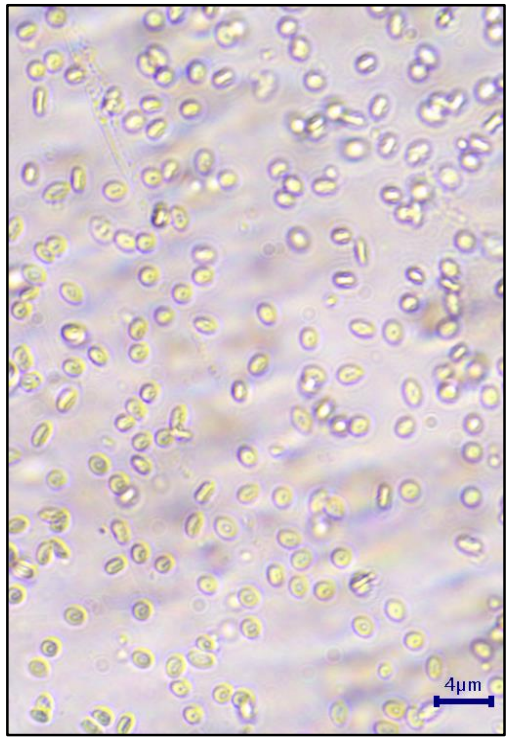
Figure 36-Indian Oil Sardine cells under 40X magnification (with Mc'coys Media)



Day 3

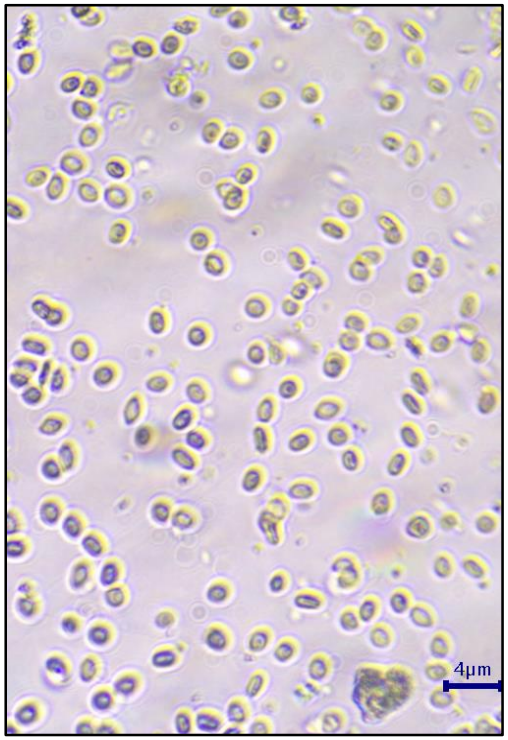


Day 2

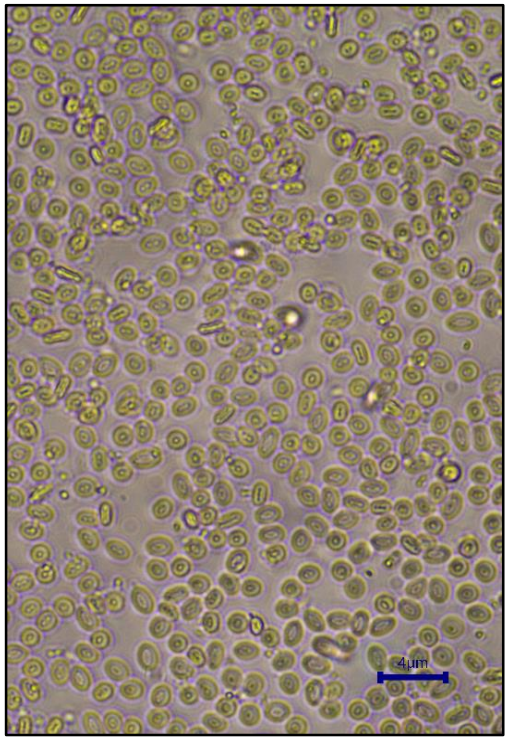


Day 1

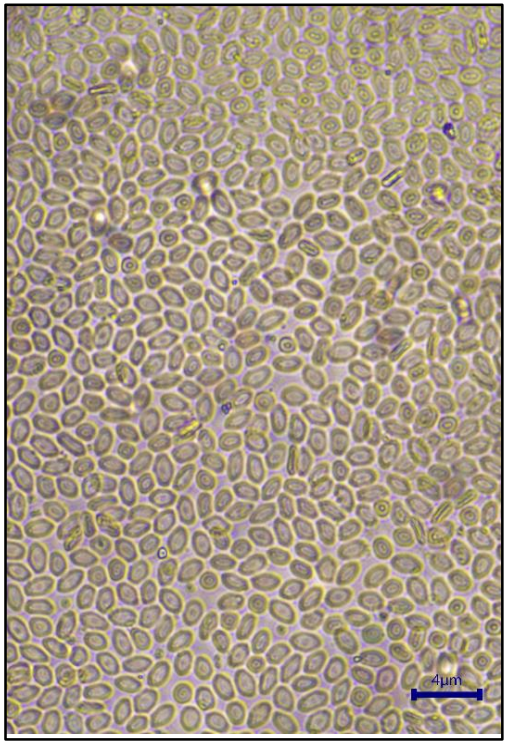
Figure 37-Flat head Grey Mullet cells under 40X magnification (with RPMI)



Day 3

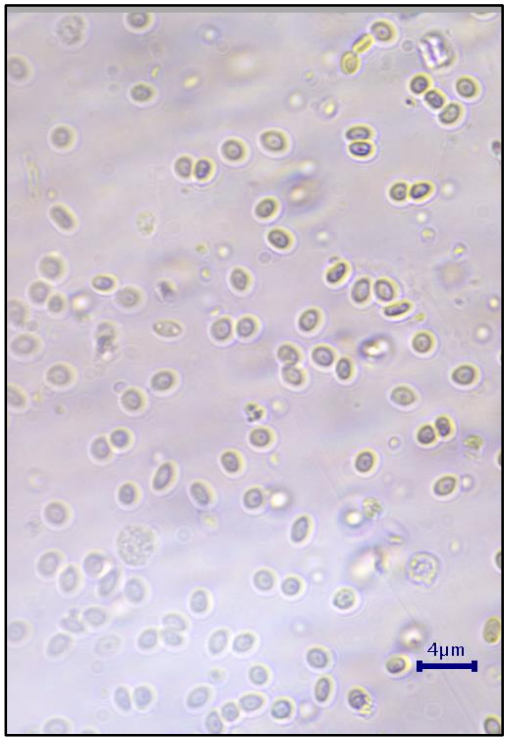


Day 2

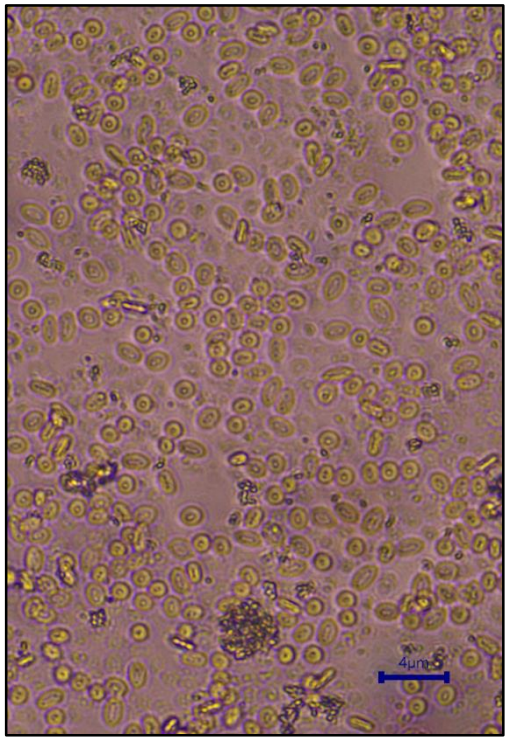


Day 1

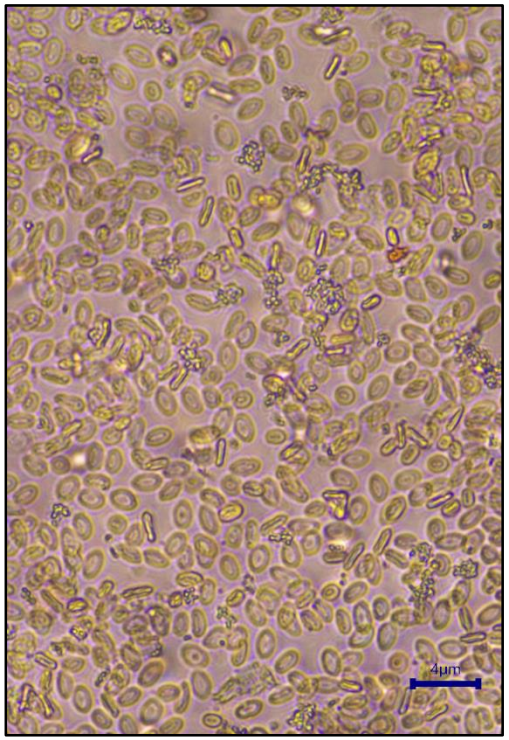
Figure 38-Flat head Grey Mullet cells under 40X magnification (with DMEM)



Day 3

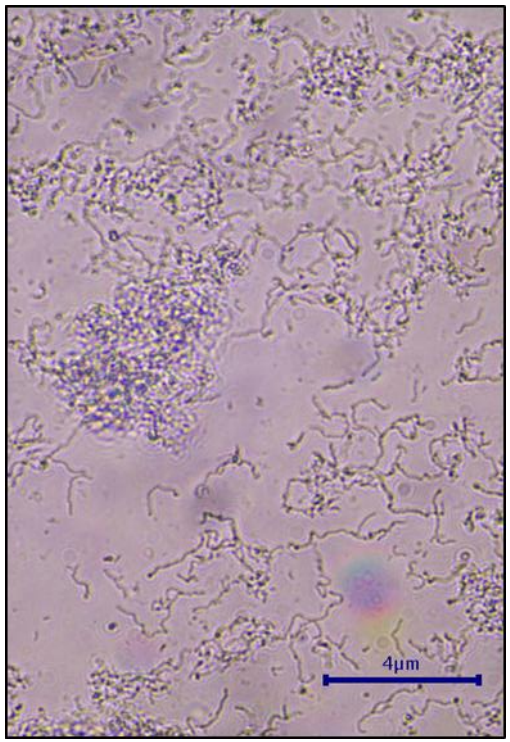


Day 2

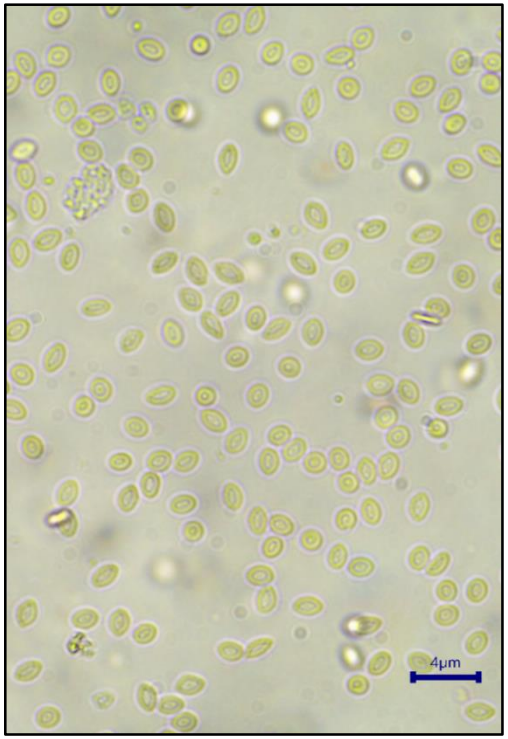


Day 1

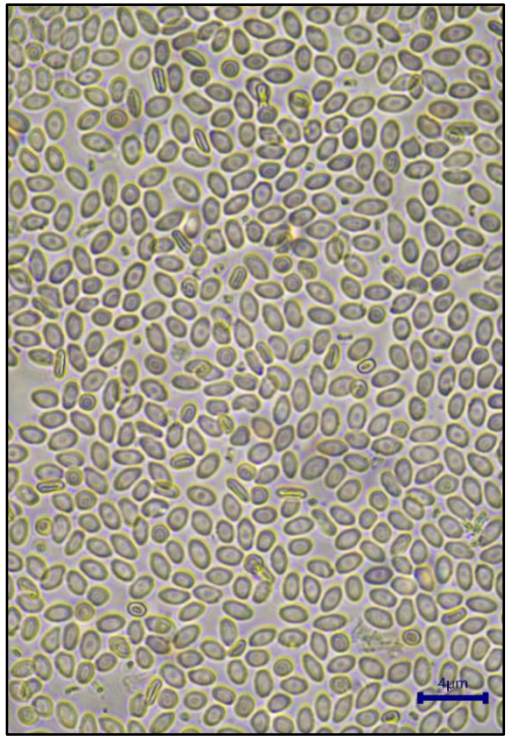
Figure 39-Flat head Grey Mullet cells under 40X magnification (with Mc'coys media)



Day 3



Day 2



Day 1

4.2 CONCLUSIONS

In the realm of cytogenetic research, the establishment of blood cell cultures from Indian Mackerel (*Rastrelliger kanagurta*), Indian Oil Sardine (*Sardinella longiceps*), and Flathead Grey Mullet (*Mugil cephalus*) represents a substantial leap forward. This study delved into the intricate genetic landscape of these ecologically and economically vital fish species, shedding light on their chromosomal features and genetic diversity.

The journey commenced with the meticulous optimization of culture conditions, a crucial step in ensuring the viability and growth of blood cell cultures. Through experimentation with various culture media compositions and temperature regimes, the study identified the most effective conditions for initiating lymphocyte cultures. The observation of metaphase within a remarkably short incubation period of 24 hours underscored the efficacy of these optimized conditions, laying a solid foundation for subsequent karyotypic analyses.

Refinement of karyotyping procedures emerged as another pivotal aspect of this study. Standardized protocols were developed to synchronize cell division, obtaining metaphase-arrested cells conducive to accurate chromosome analysis. By investigating staining techniques and imaging methods, the resolution and quality of metaphase chromosome spreads were significantly enhanced. This methodological refinement not only improved the accuracy of karyotype preparation but also paved the way for detailed characterization of chromosomal features across the studied fish species.

The heart of the study lies in the characterization of chromosomal features through karyotype analysis. Each fish species revealed its own unique genetic signature, manifested in the chromosome number and morphology observed. Indian Mackerel exhibited a diploid chromosome count of [number], with distinct banding patterns indicative of its genetic makeup. Similarly, Indian Oil Sardine and Flathead Grey Mullet displayed diploid chromosome counts of [number], each showcasing its own chromosomal arrangement and genetic traits. These findings not only

provide a glimpse into the genetic architecture of these species but also contribute to our understanding of their evolutionary history and population dynamics.

The implications of this study extend beyond the confines of the laboratory, reverberating throughout the realms of marine biodiversity and conservation. By unraveling the genetic diversity and chromosomal characteristics of these fish species, the study offers valuable insights that can inform conservation strategies and management practices in coastal ecosystems. Understanding the genetic makeup of marine populations is essential for devising effective conservation measures that safeguard the delicate balance of marine ecosystems and ensure the sustainable exploitation of marine resources.

Looking ahead, the findings of this study open up new vistas for further exploration. Future research endeavors could delve deeper into the chromosomal intricacies of these fish species, exploring additional genetic markers and genomic regions to unravel their genetic diversity comprehensively. Moreover, comparative studies across diverse populations and geographic regions could provide insights into the adaptive potential and resilience of these species in the face of environmental challenges.

In conclusion, this study represents a significant contribution to the field of cytogenetics, offering a comprehensive glimpse into the chromosomal characteristics and genetic diversity of Indian Mackerel, Indian Oil Sardine, and Flathead Grey Mullet. With its methodological innovations and insightful findings, this study not only advances our scientific understanding but also underscores the importance of genetic diversity conservation in marine ecosystems.

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