DOI https://doi.org/10.5281/zenodo.10049652#88

TWIST, 2024, Vol. 19, Issue 1, pp. 246-255

TWIST

Journal homepage: www.twistjournal.net

Evidence of Seasonal Variations in the Biochemical Composition of Subcellular Fractions in Tissues of the Freshwater Mussel *Lamellidens marginalis*

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Abstract
During our study, we investigated the changes in protein concentration, glycogen content, and total lipid content in two different natural ponds, specifically in freshwater mussels' foot, gills and digestive glands. This kind of research has never been done before. We discovered that digestive gland tissues have the highest levels of protein, glycogen, and total lipids. In both ponds, during the monsoon period (September to November 2021), we observed that digestive gland cytosol and microsome had the highest protein levels, followed by digestive gland microsome, gill cytosol and microsome, and foot cytosol and microsome. Additionally, glycogen levels were higher in gill cytosol and foot cytosol fractions in Pond 2 during the monsoon period, compared to Pond 1 in the post-monsoon period (December 2021 to February 2022). During the post-monsoon period in both ponds, total lipid content in microsome fractions of digestive gland tissue was higher than in foot cytosol and microsome fractions during the monsoon period. Our study suggests that digestive gland tissues are crucial in determining the health of *L. marginalis* as they contain the highest levels of these biochemical constituents.

Keywords
Body tissues, Freshwater pond, *Lamellidens marginalis,* Seasonality of biochemical constituents, Subcellular fractions

INTRODUCTION

In many countries across the globe, people regularly consume sea and freshwater mussels as a part of their diet. Aquaculture has emerged as an efficient industry to ensure the continuous production of these shellfish. As mussels are immobile creatures, they play a vital role in food chains and have an impact on the clarity, chemistry, and organization of water. This ultimately contributes to the functioning of the ecosystem [1]. Maintaining the quality of aquatic ecosystems is one of the biggest challenges of the 21st century. In regions with ample rainfall, ponds can form naturally, which are small bodies of water with a relatively large riparian (bank) zone and little or no deep areas.

Water pollution is a serious problem caused by various industrial effluents and human activities. These pollutants enter aquatic environments and can contain heavy metals and pesticides that are non-biodegradable, highly toxic, and have negative effects on most organisms. This is a global concern. Mussels have a mixed-function oxygenase system that is useful for monitoring the biological effects of organic pollutants. Enzyme activities can be used to assess the impact of pollution. Biomarkers such as physiological, molecular, histological, and cellular markers play a crucial role in ecotoxicology and pollution monitoring, as they can diagnose the effects of pollution and detect sublethal and chronic effects of ecological change [2]. This helps in environmental risk assessment and predicting the early detection and impact of water pollution.Biochemical changes at the organism's level help develop a reliable approach to environmental risk assessment, predicting early detection and impact of water pollution, and understanding the response of organisms to environmental pressures [3,4,5,6]. Mussels are used as bioindicators in surveillance studies worldwide due to the persistent anthropogenically induced impacts on environmental health status.

Living organisms require various nutrients, including proteins, carbohydrates, lipids (fats and oils), vitamins, and minerals for their growth and overall health. Researchers have noted that the biochemical composition of freshwater mollusks changes fortnightly and monthly [7]. Many studies have been conducted worldwide to investigate the seasonal changes in the biochemical composition of various mollusk species, to gain a better understanding of their dietary quality. These studies aim to quantify and qualify the nutrient content of different types of mollusks throughout the year.

Seasonal variations in reproductive activity and gross biochemical composition have been reported in adult mussels *Tapes decussates* and *Tapes philippinarum* [8]; biochemical constituents in *Crassostrea madrasensis* and *Perna viridis* from the Kali Estuary, Karnataka [9]. Biochemical composition of the adductor muscle, mantle, siphon, and foot in *Mercenaria mercenaria*, and *Mytilus edulis* through seasonal variations studied [10,11,12]. Some Indian researchers, on *Crassostrea cucullata* [13]; on *paphia laterisulca* [14]; on *Parreysia spp*., [15]; age, reproductive stage, and change in food concentration and temperature in marine invertebrates, qualitative and quantitative lipid changes in *Macoma balthica* [16], 14-day and monthly changes in *Lamellibrach* mollusks[7], seasonal variations in the Pacific oyster *Crassostrea gigas* [17,18,19], biochemistry, energy metabolism, growth in the scallop *Lyropecten (Nodipecten) nodosus* [20] in the *Venerid Ruditapes decussatus* [21,22] in *Charybdis Feriatus* [6]; in the oyster *Ostrea edulis* [23] found differences in biochemical composition related to the reproductive cycle of the mussels.

Biochemical indicators are highly sensitive to stressors, and the extent of biochemical changes is often related to the severity of the toxins present [1,15]. The biochemical composition of mollusks has been primarily studied to assess their nutritional status and provide additional information on their reproductive biology. During their growing season, mollusks usually accumulate carbohydrates in large quantities, which they use throughout their lives. Additionally, some mussel species use proteins as an energy store [24,25].

A study on cadmium chloride and summer-induced changes in the freshwater mussel *L. marginalis conducted* [26], while [27] examined the effects of summer on the mussel *Parreysia rugosa* and [28] investigated the sublethal effects of summer on the tissue composition of *L. marginalis*. Other studies have focused on seasonal biochemical changes in different body tissues of freshwater mollusks, such as *Mytilus viridis* from the Godavari River near Aurangabad [28], and the lipid composition of egg and adductor muscle in giant clams *P. magellanicus* [29]. The hepatopancreas is the primary reserve and detoxification organ for foreign matter in mollusks [30].

A study was conducted on the freshwater mussel *L. marginalis* to establish a baseline understanding of the distribution of biochemical components in its tissues and subcellular fractions. The literature review reveals insufficient data on the seasonal variation of biochemical components in subcellular fractions of different tissues such as the foot, gills, and digestive gland of freshwater mussels. To fill this knowledge gap, a preliminary study was conducted on freshwater mussels in natural ponds located in Chinna Kodunthurai and Thiruthiyamalai, near Musiri Taluk, Tiruchirappalli district, as information on the tissue and subcellular distribution of biochemical constituents is currently limited.

MATERIALS AND METHODS

Study area and data collection

The research area is situated in Musiri Taluk, Tiruchirappalli District, Tamil Nadu, India, as shown in Figure-1. In this area, there are two ponds: Chinna Kodunthurai Pond-I (Figure-2) is surrounded by paddy fields that receive water during the rainy season and from the nearby paddy fields. Thiruthiyamalai Pond II (Figure-3) is located among rocks, also receives water during the rainy season, and is used by humans and cattle. From September 2021 to February 2022, freshwater mussels of the species *Lamellidens marginalis* (measuring 6-7 cm in length and weighing 25-27 g) (Lamarck) were collected from Pond-I (Chinnakodunthurai) and Pond-II (Thiruthiyamalai) in Musiri Taluk, Tiruchirappalli district (Tamilnadu, India). After collection, the mussels were taken to the laboratory, cleaned, and placed in cement tanks for 24 hours to acclimate before analysis.

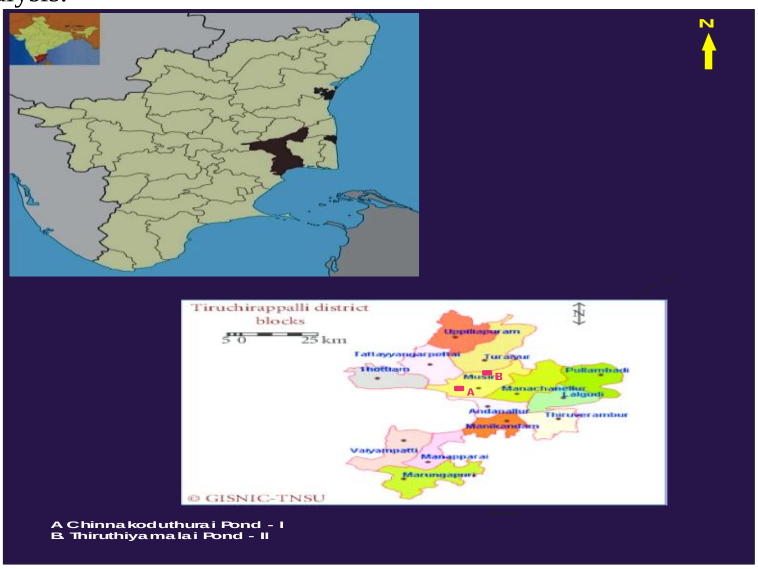

Fig. 1 The geographical location of the study is Musiri Taluk, Tiruchirappalli District, Tamilnadu State, India

Fig. 2 Chinnakodunthurai fresh water pond-I

Fig. 3 Thiruthiyamalai fresh water pond- II

Preparation of Sub-cellular fractions

Subcellular fractions, namely cytosol, and microsomes, were prepared from the gill, foot, and digestive gland tissues of freshwater mussels, using the method described [1]. The entire manufacturing process was carried out at a temperature of 4°C. The pooled tissues were homogenized in a solution containing 1 g/4 ml of 20 mM Tris-HCl (pH 7.6), 0.25 M sucrose, 0.15 M KCl, 1 mM EDTA, 1 mM DTT, and 100 1M PMSF. All manufacturing procedures were performed at 4°C. The homogenized samples were first centrifuged at 600 g for 10 minutes to sediment cell nuclei and cell fragments. Then, without transferring these fragments, the samples were recentrifuged at 12,000 g for 45 minutes. As a result, the supernatant was collected and used as a mitochondrial fraction. The resulting pellet was resuspended in homogenization buffer and centrifuged at 100,000 g for 90 minutes, and then the supernatant was collected and used as the cytosolic fraction. A resuspension buffer of 20 mM Tris (pH 7.6), 1 mM dithiothreitol, 1 mM EDTA, and 20% v/v glycerol was added to the remaining pellet. This resuspended pellet was used as the microsomal fraction

Biochemical Analysis

The techniques developed by [31,32,33] were used to estimate carbohydrates, proteins, and lipids respectively.

Statistical analysis

For each parameter, three determinations were made and the SEM mean (standard error of the mean) was calculated statistically. Linear correlation and student's t-test was used to assess differences between datasets, and a significance level of $P<0.05$ was taken as indicating a significant difference .

RESULTS AND DISCUSSION

Protein

The figure 4-5 shows seasonal changes in protein content in the cytosol and microsomes of freshwater mussels. The protein content is expressed as mg per g of dry weight, and the linear correlation between values is $y=14.771x-1.8433, R_2=0.866$ in pond-I and the linear correlation between values is $y=16.443x+45.861, R_2=0.6826$ with a significance level of $P<0.05$ in pond-II. During the test period, the protein content showed minor fluctuations and reached its highest level in the cytosol of the digestive gland in September 2021 in ponds I and II (141.42±0.11 mg/g and 138.43±0.11 mg/g, respectively). It fell to the lowest level (21.17±0.1 mg/g) in the microsomes of foot tissues in pond II and in the cytosol of the foot (20.16±0.01 mg/g) in December 2021 in pond I. The study explains the increase and decrease in protein levels in relation to the reproductive and environmental factors that affect the mussels. Similar results were observed by several researchers [34,35,36,37,15,28,38,6,39]. These studies showed that the decline in food availability and the increase in mean summer temperature and other external factors can decrease the protein content in mussels. This could be due to the animal's need to diversify its energy sources to cope with environmental stress. Similar results were also observed in

Lamellidens marginalis of the Godavari River [35]. The availability of food is closely related to the energy needed for growth, energy reserves during gametogenesis, and reproductive development. The maximum protein content (29%) was observed in November 2021 in the cytosol of the digestive gland in both ponds and the linear correlation between values is $y=17.755x+39.265, R_2=0.7342$ with a significance level of $P<0.05$ (Fig. 6), while the minimum was observed in November 2021 in the cytosol and microsomes of the gill (7% in pond I and pond II, respectively) and the linear correlation between values is $y=16.443x+45.861, R_2=0.6826$ with a significance level of $P<0.05$ (Fig. 7). Proteins are the major biochemical constituents of mussel tissue, varying between 63.1% in December and 72.3% in August [40].

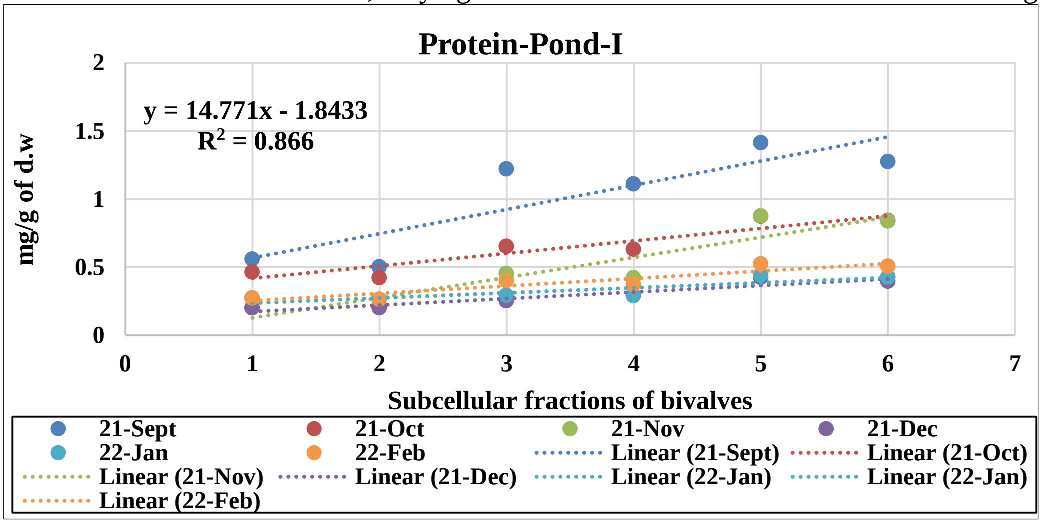

Fig. 4 Monthly variation of total protein (mg/g) in subcellular fractions of *L. marginalis* from Sep-2021 to Feb-2022 in Pond-I. Data are presented as mean SEM (standard error of the mean) N=3

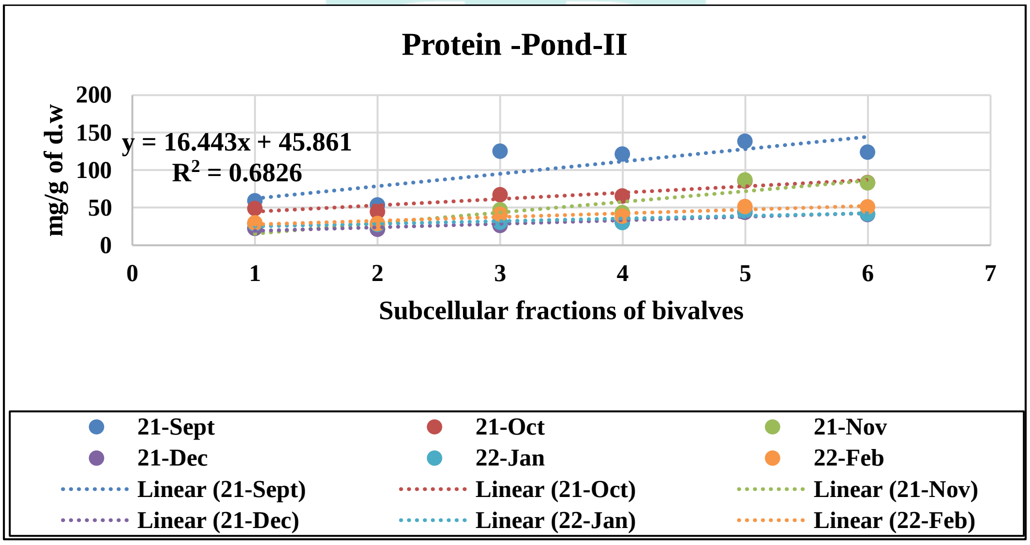

Fig. 5 Monthly variation of total protein (mg/g) in subcellular fractions of *L. marginalis* from Sep-2021 to Feb-2022 in Pond-II. Data are presented as mean SEM (standard error of the mean) N=3

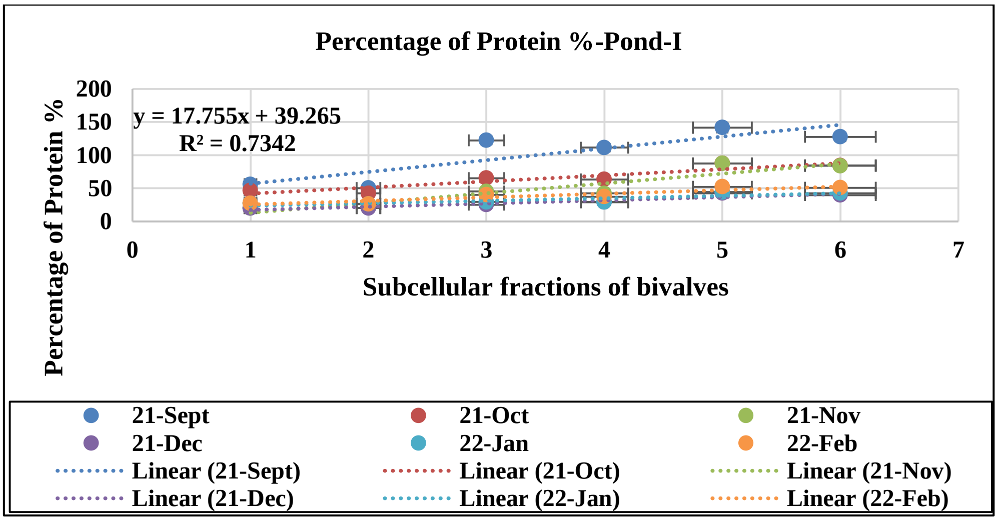

Fig. 6 Monthly percentage (%) variation of protein (mg/g) in subcellular fractions of *L. marginalis* from Sep-2021 to Feb-2022 in Pond-I

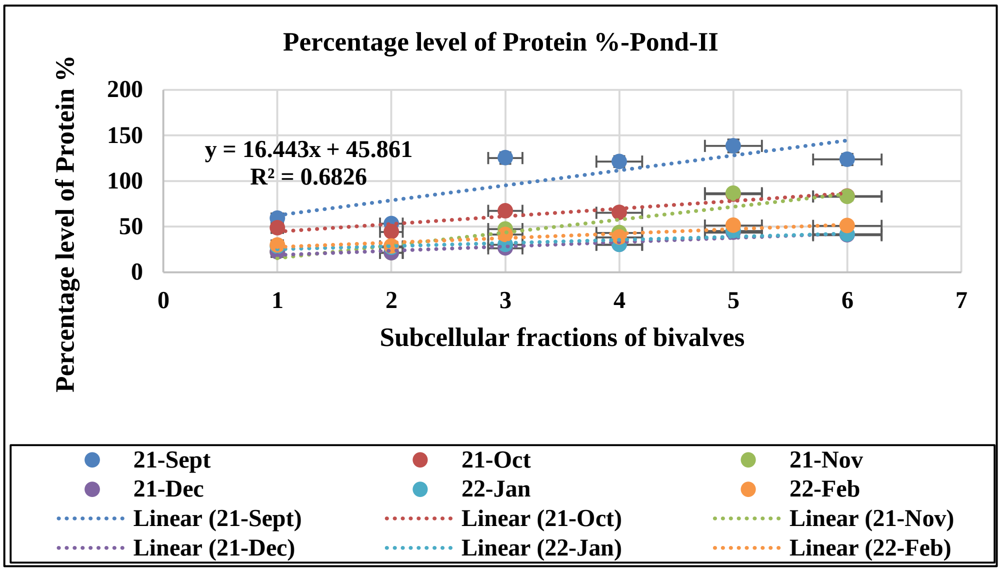

Fig. 7 Monthly percentage (%) variation of protein (mg/g) in subcellular fractions of *L. marginalis* from Sep-2021 to Feb-2022 in Pond-II

Glycogen

The figure 8-9 in this study shows the seasonal changes in glycogen content in freshwater mussels, measured in mg per g of dried meat weight and the linear correlation between values is y=1.9114x+41.073,R_2=0.8566 in pond-I and the linear correlation between values is y=1.7343x+42.353861,R_2=0.8002 with a significance level of $P<0.05$ in pond-II. The glycogen content remained relatively stable during the study period, with the highest levels found in the cytosols of the digestive gland (53.22±0.96 mg/g) and microsomes of the digestive gland (52.33±0.96 mg/g) in February 2022, in ponds II and I, respectively. The lowest levels were found in the cytosols of the gill (30.19±0.05 mg/g) and microsomes of the gill (29.19±0.01 mg/g) in December 2021, in ponds II and I, respectively. The digestive gland had the highest glycogen content, followed by the gills and the foot, in both ponds. Glycogen levels increased gradually from winter to summer and peaked in the digestive gland tissues during the summer season. This is consistent with previous studies that have shown glycogen levels decreasing after dormancy, reaching minimum levels during spawning, and recovering after spawning. Spawning of mussels occurs during the monsoon season, which causes a depletion of protein and lipid content. After spawning, the mussels begin to accumulate and store carbohydrates in their tissues, with glycogen being the most important nutritional element as it is the energy source for the anaerobic metabolism of many mussels. The maximum glycogen (20%) was reached in January 2022 in the cytosol and microsomes of the digestive gland in both ponds and the linear correlation between values is y=1.9114x+41.073,R_2=0.8566 in pond-I (Fig. 10), and the minimum was reached in the cytosol of the gill at 14% in November 2021, January 2022 in pond-I, and January 2022 and the linear correlation between values is y=1.7343x+42.353861,R_2=0.8002 with a significance level of $P<0.05$ in pond-II (Fig. 11). The glycogen content ranged from 9.5% from August to 20.9% in December. Glycogen content influenced breeding behavior and gonad development in general [40].In bivalves, gonad development may involve the metabolic conversion of glycogen to lipids [12].Glycogen content can influence breeding behavior and gonad development in bivalves, as gonad development may involve the metabolic conversion of glycogen to lipid.The following researchers made similar observations [17,4,20, 8, 28,38,6,39].

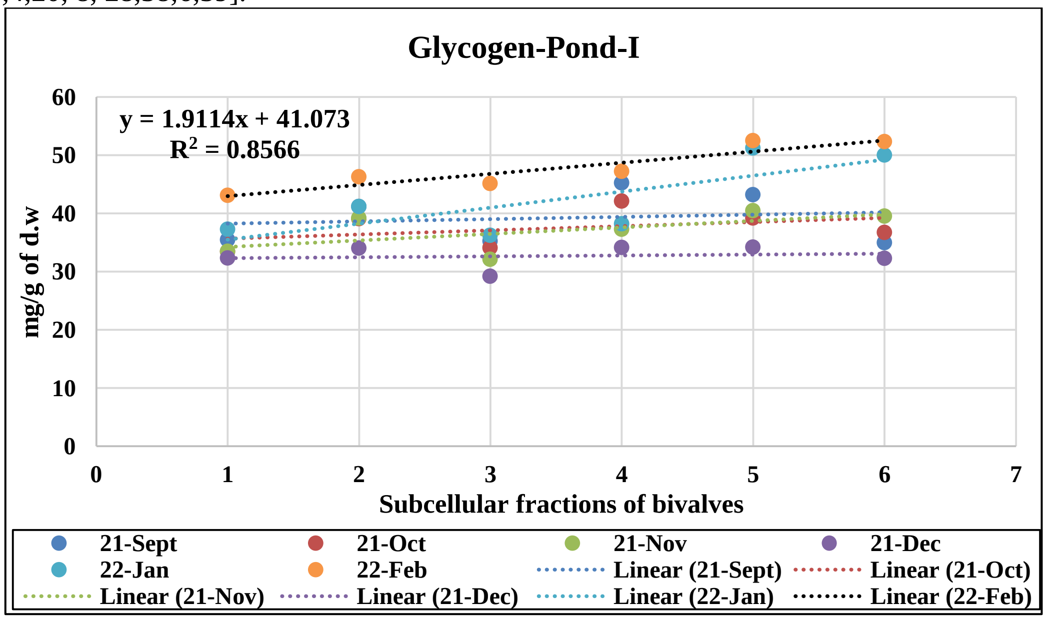

Fig. 8 Monthly variation of total glycogen (mg/g) in subcellular fractions of *L. marginalis* from Sep-2021 to Feb-2022 in Pond-I. Data are presented as mean SEM (standard error of the mean) N=3

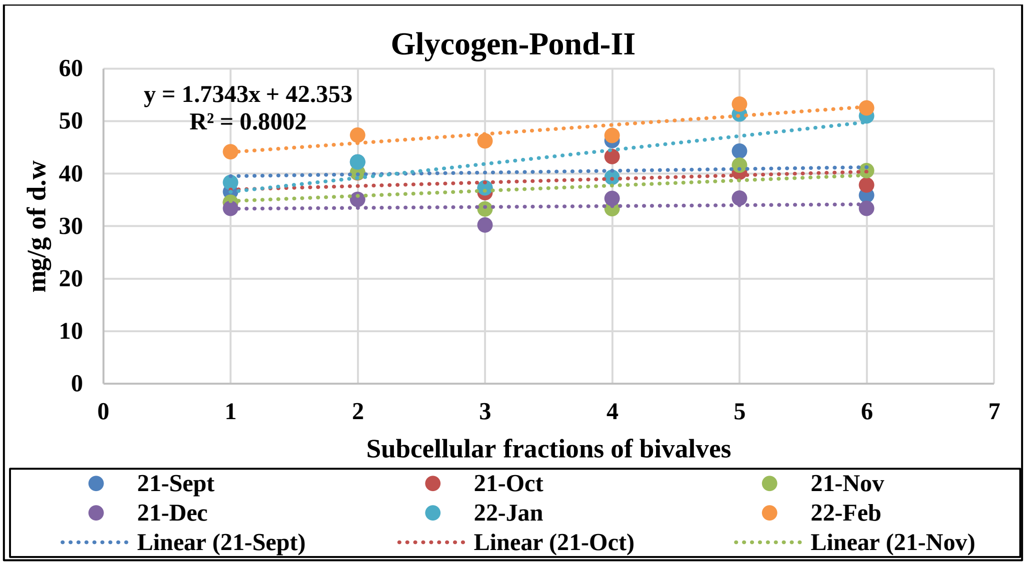

Fig. 9 Monthly variation of total glycogen (mg/g) in subcellular fractions of L. marginalis from Sep-2021 to Feb-2022 in Pond-II. Data are presented as mean SEM (standard error of the mean) N=3

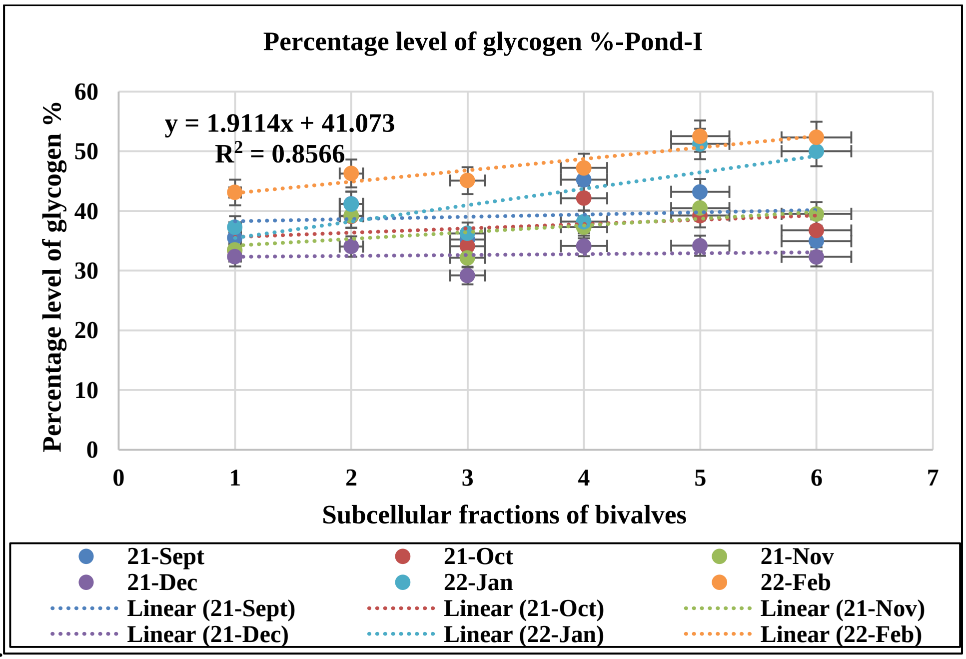

Fig. 10 Monthly percentage (%)variation of total glycogen (mg/g) in subcellular fractions of *L. marginalis* from Sep-2021 to Feb-2022 in Pond-I

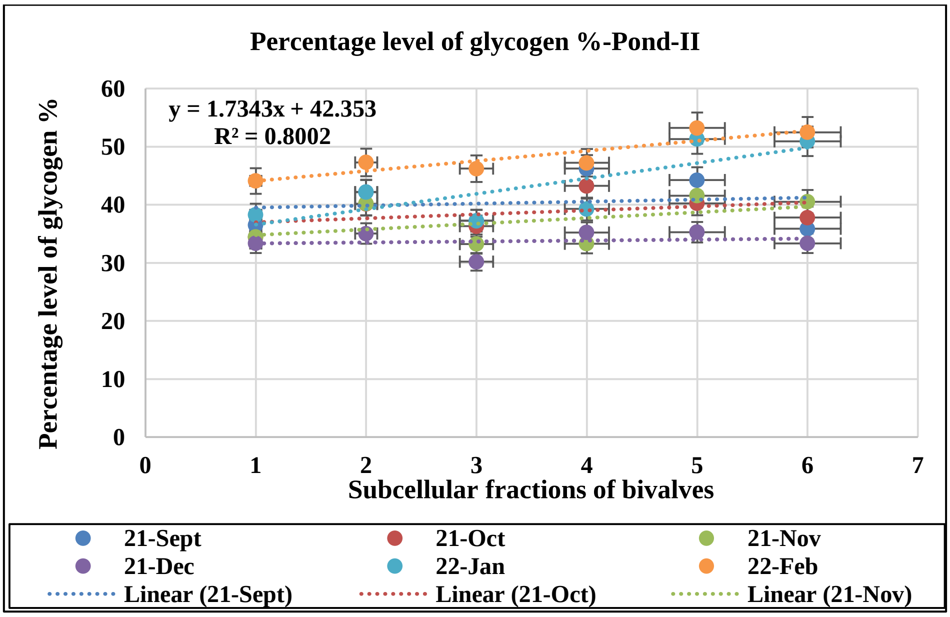

Fig. 11 Monthly percentage (%)variation of total glycogen (mg/g) in subcellular fractions of *L. marginalis* from Sep-2021 to Feb-2022 in Pond-II

Lipids

Seasonal variations in lipid content in the cytosol and microsomes of freshwater mussels are shown in Fig 12-13, expressed as mg per g of dry meat weight and the linear correlation between values is y=1.2591x+8.952,R_2=0.8043 in pond-I and the linear correlation between values is y=1.344x+10.057,R_2=0.7621 with a significance level of $P<0.05$ in pond-II. The lipid content was subject to only a few fluctuations during the test period and reached a maximum in September 2021 in pond II and I (9.83± 0.01 mg/g);(8.53±0.01 mg/g) in cytosols of the foot tissue and was noticeable a minimum of (12.90±0.29 mg/g); (1.23±0.04 mg/g) digestive gland microsomes in December-2021 in Pond-I and November in Pond-II. The present research confirms the importance of lipids in energy metabolism. Lipids are a crucial component of the reproductive process, which is why gonads maintain lipid storage. Several recent observations have also confirmed these results [18,9,15,28,38,6,39]. For instance, *Parreysia spp*. in Tungabhadra River, Karnataka, exhibited high lipid levels during the summer season due to exposure of the coat and foot to high temperatures [28,38,6,39]. In *L. marginalis* from Pravara River in Maharashtra, lipid variations were observed in foot tissue during post-monsoon, where as here the maximum lipid fraction (34%) was reached in November 2021 in the cytosol of the foot in pond-II (Fig. 14) and the minimum was reached in November 2021 in the microsomes of the digestive gland at 5% in pond-II (Fig. 15). A lipid response of 5.6% was observed in the bivalve *Ruditapes decussatus* throughout the year, and glycogen may be converted to lipid during gonad development [40,12].

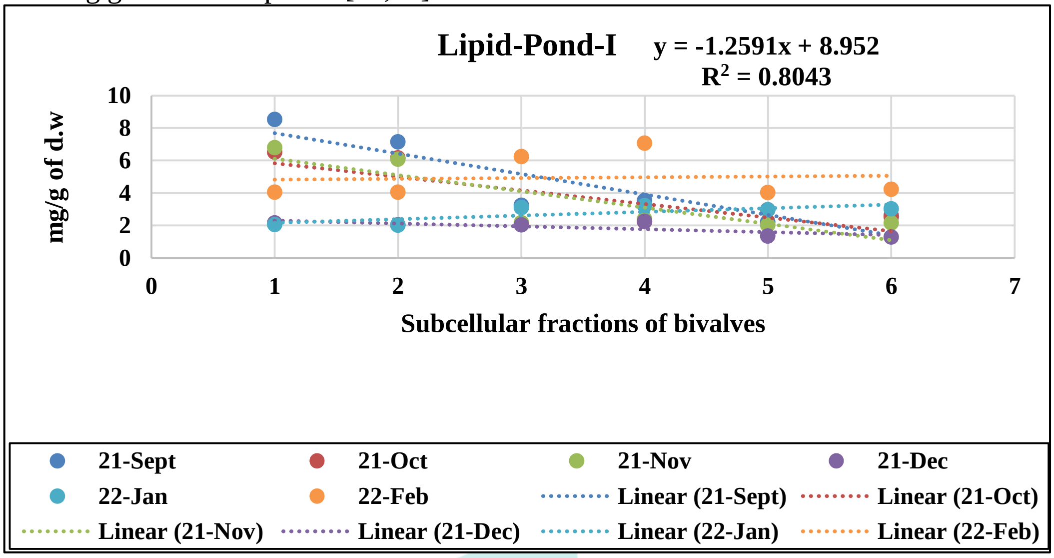

Fig. 12 Monthly variation of total lipid (mg/g) in subcellular fractions of *L. marginalis* from Sep-2021 to Feb-2022 in Pond-I. Data are presented as mean SEM (standard error of the mean) N=3

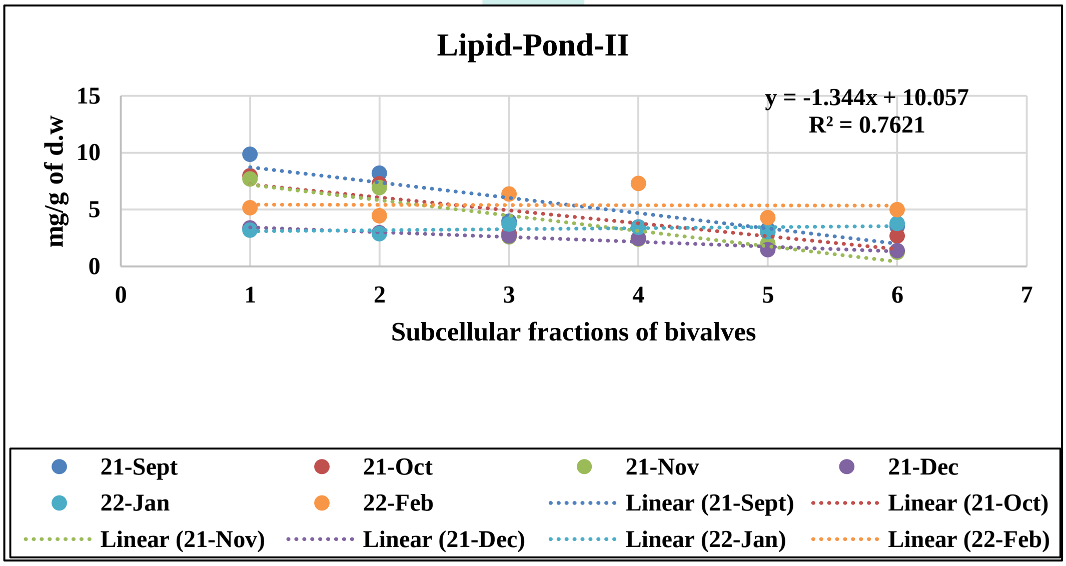

Fig. 13 Monthly variation of total lipid (mg/g) in subcellular fractions of *L. marginalis* from Sep-2021 to Feb-2022 in Pond-II.Data are presented as mean SEM (standard error of the mean) N=3

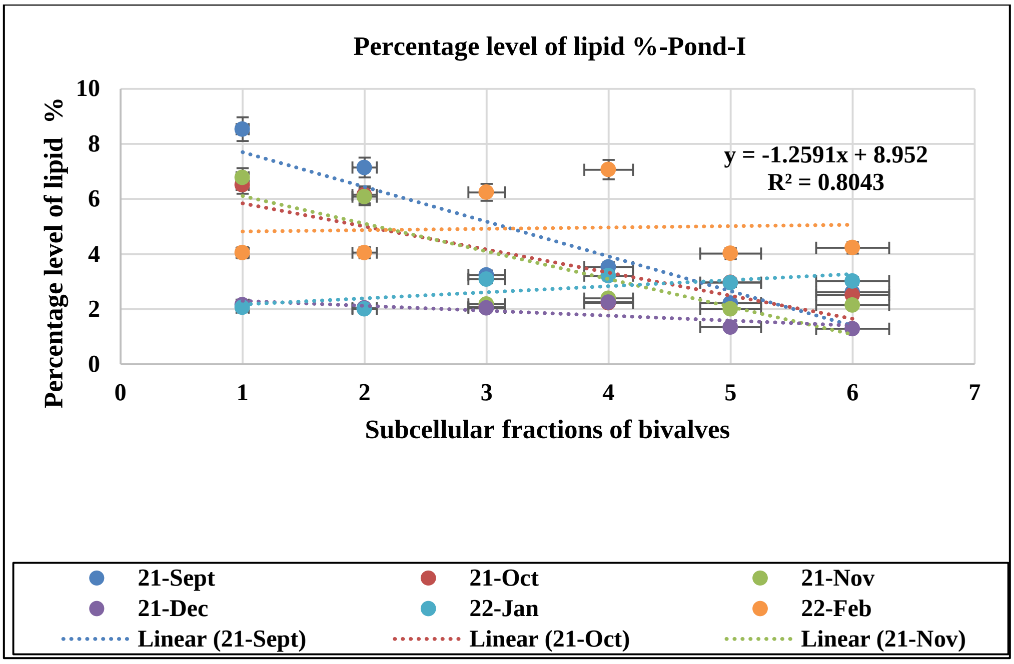

Fig. 14 Monthly percentage (%)variation of total lipid (mg/g) in subcellular fractions of *L. marginalis* from Sep-2021 to Feb-2022 in Pond-I

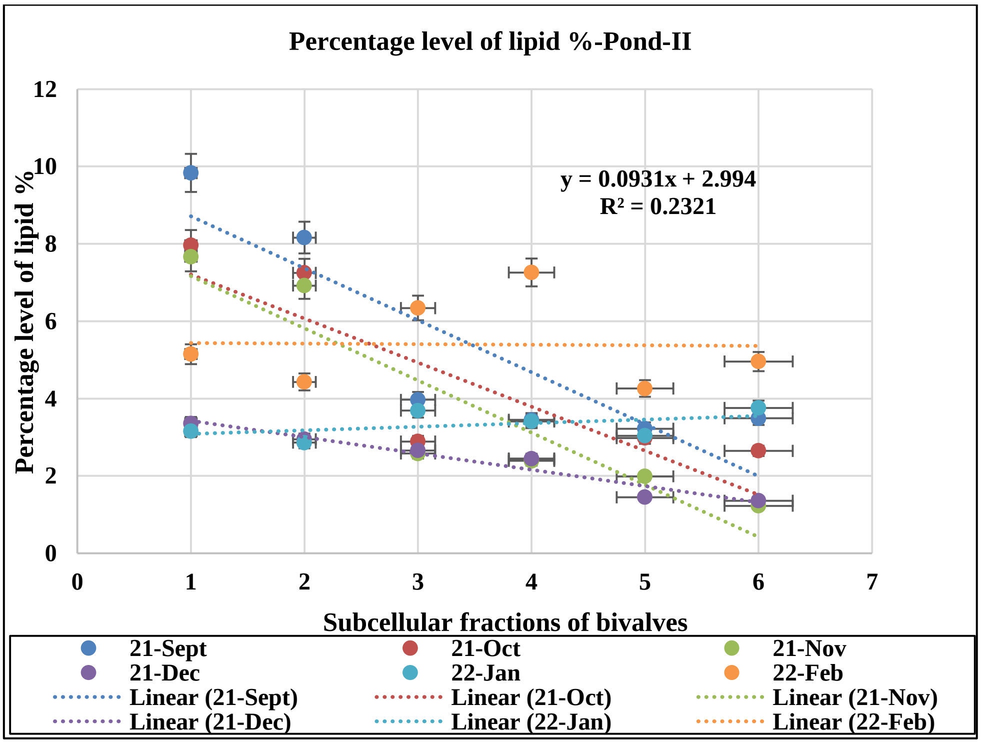

Fig. 15 Monthly percentage (%)variation of total lipid (mg/g) in subcellular fractions of *L. marginalis* from Sep-2021 to Feb-2022 in Pond-II

The latest research indicates that the MFO (mixed function oxidase) system is primarily found in the digestive gland of freshwater mussels and is largely or entirely membrane-bound in the endoplasmic reticulum. This conclusion is based on enzyme studies conducted on the freshwater mussel *Lamellidens marginalis* [4,5,3]. The digestive gland is a crucial site for the uptake of environmental toxins and temperature [10]. The reproductive cycle primarily influences protein variation, while a variety of factors, including salinity, temperature, day length, and the density of the surrounding medium, determine the food availability cycle. Lipid accumulates in the developing gonads and is expended during spawning, while glycogen is expended in the development of gametes. Therefore, the study confirms that cytosolic, microsomal subcellular fractions/components of biochemical constituents (proteins, glycogen, and lipids) are primarily found in the digestive glandular tissue of freshwater mussels, followed by gills and foot. This confirms the importance of the seasonality of biochemical components in *L. marginalis*. A new approach to the seasonality of biochemical components in the freshwater mussel *Lamellidens marginalis* is likely. The research opens up the possibility of developing it as a specific index of seasonal biochemical components and biological impacts, and future studies can now be focused on achieving this goal.

CONCLUSION

The digestive gland, also known as the digestive diverticulum, is believed to have an important role in the digestion process (mucoproteins) and reproduction of mussels. Its microsomal and cytosolic fractions are crucial in maintaining the physiological and reproductive phases of mussels. Biochemical components make compensatory adjustments to changes in environmental conditions for both energy gain and energy loss components. The biochemical differences between tissues of subcellular fractions reflect morphological, evolutionary, and functional closeness, and illustrate the distinct role each organ plays in the organism, despite other sources of variation such as diet and sex. Moreover, the biochemical compositions of tissues such as the foot, gills, and digestive gland can reflect the timing of reproduction and physiological status, showing the composition of body tissues. This research confirms that the foot, gill, and digestive glands (cytosol, microsomes) of the freshwater mussel *L. marginalis* can be used as a biomarker to assess the animal's health status during recultivation.

FUNDING INFORMATION

This research did not receive any specific grants from funding agencies in the public, commercial, or not-for-profit sectors.

DECLARATION OF CONFLICT

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

ACKNOWLEDGMENTS

The authors appreciate the support of the Head, P.G. and Research Department of Zoology, Arignar Anna Govt Arts College, Musiri-621 211, affiliated with Bharathidasan University Tamilnadu, India to carry out this work.

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