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Modeling the Effect of Starvation on Phospholipid Content in *Anabas testudineus* (Climbing Perch) Bloch: An Integration Approach

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ABSTRACT
Phospholipids are the structural lipids, which play a major role in stabilizing and integrating the bio membranes. They are important as signaling molecules, and maintain the cell homeostasis, but are not looked upon as potential energy substrates. Not many studies, have been conducted, to learn the role of phospholipids, as energy reserves, during stressful conditions such as starvation. Apart from being the energy substrates, for the starving animal, their, role as stabilizers to maintain the appropriate cell structure seems to be much more important for survival and to overcome the starvation stress. To understand, these aspects, the present study has been attempted. Anabas testudineus, a sturdy, fresh water fish, from local waters, was selected and subjected to short term (15 days) and long term (60days), laboratory starvation. Six tissues, such as liver, kidney, accessory respiratory organ, brain, pectoral and lateral line muscle, were selected for this study and phospholipids were estimated. Student "T" test was performed, to distinguish the level of significance of the result. Integration was used to calculate the phospholipids over time, referred to as the area under the curve, AUC (t=0days to t= 60days). This study presented, an amalgamation of elevation and decline of phospholipids, in the selected tissues. The study results, illustrate the dual attributes of phospholipids, as efficient energy sources and also as effective stabilizers of bio membranes.

KEYWORDS: *Phospholipids, Anabas testudineus, bio membranes, starvation, structural lipids, fasting*

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INTRODUCTION

Majority of fish undergo periods of natural starvation because of seasonal fluctuations in food supply or aestivation or due to non-feeding spawning migration. Many species of teleosts demonstrate the ability to withstand prolonged periods of starvation (Larsson & Lewander, 1973, Loughna & Goldspink, 1984, Machado, Garofalo, Roselino Kettelhut& Migliorh, 1988). Irrespective of particular environmental requirement of individual species, mobilization of endogenous energy sources would be expected to proceed such that energy requirements are met in the most efficient manner (Jurss, Bittorf& Volker, 1986; Sheridan &

Mommsen, 1991).Starving animal, may depend and utilize, either, carbohydrates, lipids and proteins or all of them, in a fluctuating manner. (Dave Goran, Maj-Lis Johansson - Sjobeck, Ake Larsson, Kerstin Lewander & Ulf Lidman,1975). It is well known that lipids are the major energy sources of fish (Bell and Tocher,1989) and this is because, lipid is a far superior stored fuel from gravimetric view point and the energy obtained by lipid utilization in approx 9.4cal/gm of pure lipids (Cahill, 1986). Lipid utilization may be as triglycerides or as structural lipids. There are instances, where in structural lipids, have been utilized by the fish, during starvation, as seen in *Pleuronectus platessa*, and *Anguilla anguilla* which when starved for 12 weeks, showed, the utilization of both Cholesterol and phospholipids. (Love,1980; Dave,et.al.,1975).Apart from, phospholipids, being used as energy substrates, there are evidences, where in Phospholipids, are also known to have an impact upon, growth, deformity, resistance against stress and also upon survival status, of an organism. (Kanazawa, Teshima & M. Sakamoto, 1985; Koven, Parra, Kolkovski, & Tandler 1998; Cahu, Infante & Barbosa, 2003). Recent studies, by Tina Izard and David T. Brown, 2016) have shown, that phospholipids,(inositol phospholipids),are crucial elements, necessary for attachment of viniculin protein, which is a major component essential, for cell adhesion, migration and motility of a cell, thus highlighting the importance of phospholipids as vital components of cell membranes. Based upon these studies, it is quite evident that phospholipids seem to play a very crucial role, in survival, growth and also give resistance against stress. **Therefore we hypothesize that phospholipids may be the key players, during starvation, as energy molecules, and also act as stabilizers for maintaining the compactness of bio membranes.** The objective of the present starvation study on *anabas,* is twofold. Firstly to understand the role of phospholipids, as energy sources, for starving animal, and secondly to understand if they can act as stabilizers to maintain the integrity of bio membranes. In this regard, we have selected a sturdy fresh water, fish, *anabas testudineus*, commonly known as climbing perch, available from local waters of Andhra Pradesh. The fish was subjected to short term and long term laboratory starvation, and phospholipids were estimated accordingly from six selected tissues.

MATERIAL AND METHOD

Collection of the fish

Anabas testudineus is a sturdy fresh water teleost fish, available from local waters of coastal Andhra Pradesh, (INDIA). It is a larvicidal fish, and can be identified by the presence of black colored spot at the base of caudal fin and another spot at the end of opercle. Fish weighing of 20-25gm were obtained from catchment area, Kolleru lake of *Eluru*. (AP). Care was taken to ensure quick transport to the laboratory. Overcrowding was avoided during packing to minimize the mortality rate. They were carefully transferred into Durex storage tank of capacity 500 litres, made of material corrosive resistant polypropylene. The closed plastic lid of the tank was replaced by a grill lid made of iron. This helped in proper ventilation and aeration of the tank. Fish which were injured or dead were removed from the tank from time to time. Disinfectant ($KMnO_4$) was used to avoid infection. They were given boiled egg, rice bran meal and commercial fish feed *ad. libitum.* Any leftover feed and fecal matter were removed daily. Water in the tank was changed every day. Fish were brought to the laboratory and sufficient time was allowed for acclimatization. Experimentation was done thereafter. Fish measuring about 3-4inches in length and in the same range of weight were selected carefully. They were grouped together and kept in circular tubs made of plastic. The mouth of these tubs was covered with fine mesh and appropriate by placed such that they were properly ventilated and well aerated. Two types of experimental set up were designed. In the first set up, fish were allowed to starve for 15 days and parallel control was also maintained. The control animals were fed regularly both in the morning and evening. On the 16th day both experimental and control animals were sacrificed by a concussion, and the tissues were removed for biochemical analysis. The second experimental set up consisted of fishes which were allowed to starve for 60 days (two months) (long term). Experimental group and a corresponding

control group was maintained. Control group was fed regularly as in the case of short term. On the 61st day, the animals were sacrificed, for the experimentation. Six animals in the control and six in the experimental group (starved) were killed by concussion and the tissues were analyzed. The tissues selected for the experimentation were liver, kidney, brain, pectoral muscle, lateral line muscle and accessory respiratory organ. Phospholipids were estimated by the method of Raghuramulu, Madhavan Nair, Kalyana Sundram, (1983).

Principle: The organic phospholipid phosphorus in converted into organic phosphorus which reacts with ammonium molybdate to form phosphomolybdic acid which on reduction and reaction with ANSA [Amino 2 naphthol 4 sulfuric acid] forms a stable blue color.

Procedure: A known quantity of tissue homogenate was prepared using mixture of 3:1 ethanol ether. Evaporate lipid fractions to dryness and 0.5 ml of 10 NH_2SO_4 was added. The samples were incubated in an oven of temperature 150-160^{0}C for three hours. Two drops of fuming HNO_3 were added and the solutions were returned to the oven for at least 1½ hour more to complete the combustion. The samples were removed and cooled. Ammonium molybdate solution of 4.6 ml was added, followed by 0.2 ml of ANSA. The tubes were covered with marbles and heated for 7 mints in boiling water bath. The color developed was read at 660nm with the use of a Beckman model DB Spectrophotometer. A working Standard was prepared using mono potassium phosphate dissolved in $10NH_2So_4$. The amount of phospholipids was expressed as mg of phosphate/gm wt. of tissue.

Note: The color produced was proportional to the concentration of phosphorus up to 1.5 µmoles in the reaction mixture. These values may be expressed as the phospholipids (lecithin) by multiplying by a factor of 25.

RESULTS

Short term starvation stress of *Anabas*, lead a decline in the phospholipid concentration in tissues like accessory respiratory organ, pectoral muscle and lateral line muscle. Brain did not show any change i.e. the phospholipid concentration remained the same in both control and experimental animals. Liver and kidney showed a significant increase. Liver showed an increase of 163.4% ($P<0.001$). Kidney showed an increase of 35.6% ($P < 0.02$) (**Table-I and Fig-1**) Brain tissue showed no change. The decrease seen in Accessory respiratory organ was found to be 55.7% ($P<0.01$) (**Table-II and Fig-2**) Pectoral muscle showed a decline of 43.6% ($P<0.01$) and the decrease seen in lateral the muscle was found to be 36.2% ($P<0.01$) (**Table-III, Fig-3**) Long term starvation of *Anabas*, resulted in increase of phospholipids in tissues like liver, kidney brain and pectoral muscle. Other tissues like accessory respiratory organ and lateral line muscle showed a depletion in the phospholipid content. To determine the decline or elevation, student "t test was performed and significance level was calculated. The increase in liver was found to be 87% ($P<0.001$). Kidney showed an increase of 31.4% ($P<0.10$). Brain showed an increase of 48.1% ($P<0.05$). The increase in pectoral muscle was 23.6% (NS). Accessory respiratory organ showed a decrease of 24.3% ($P<0.001$) and the decrease in lateral line muscle was found to be 42% ($P<0.01$).

Table 1: Phospholipid Levels in Liver and Kidney tissues during Short Term and Long Term starvation in *A. testudineus*

S. No.	Tissue analyzed	Short Term (15 Days)		Long Term (60 Days)	
		Control	Expt.	Control	Expt.
1.	Liver	517 SE = ± 25.8	1362*** SE = ± 60	762 SE = ± 32.963	1425*** SE = ± 53.136
		% Variation = +163.44		% Variation = +87	
2.	Kidney	235.8 SE = ± 17.607	319** SE = ± 14	242.833 SE = ± 12.7	319.166* SE = ± 77.805
		% Variation = +35.623		% Variation = +31.434	

Values expressed in mg of phospholipid / gm wt. of tissue
Each value is mean of SE ± of 6 individual observations
P < 0.10*, P < 0.02, P < 0.001*****

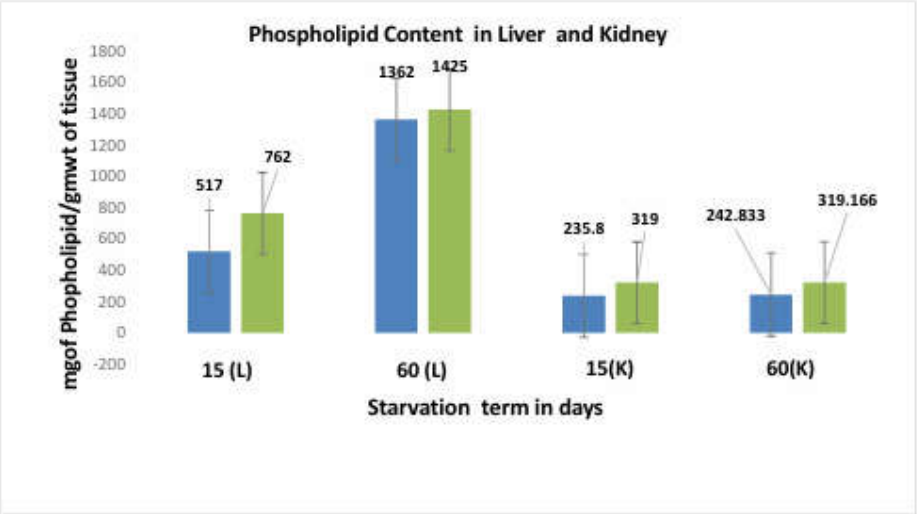

Figure 1: Phospholipid Content in Liver and Kidney

Table 2: Phospholipid Levels in Brain and Accessory Respiratory Organ during Short Term and Long Term starvation in *A.testudineus*

S. No.	Tissue analyzed	Short Term (15 Days)		Long Term (60 Days)	
		Control	Expt.	Control	Expt.
1.	Brain	180.16	180.16	187	277.333**
		SE = ± 22.2	SE = ± 22.2	SE = ± 14.3	SE = ± 23.2
		No Change		% Variation = +48.128	
2.	Accessory Respiratory Organ	228.8 SE = ± 17.8	101.16* SE = ± 8.5	201.166 SE = ± 27.3	152.666*** SE = ± 20.5
		% Variation = -55.786		% Variation = -24.378	

Values expressed in mg of phospholipid / gm wt. of tissue
Each value is mean of SE ± of 6 individual observations
P < 0.01*, P < 0.05, P < 0.001*****

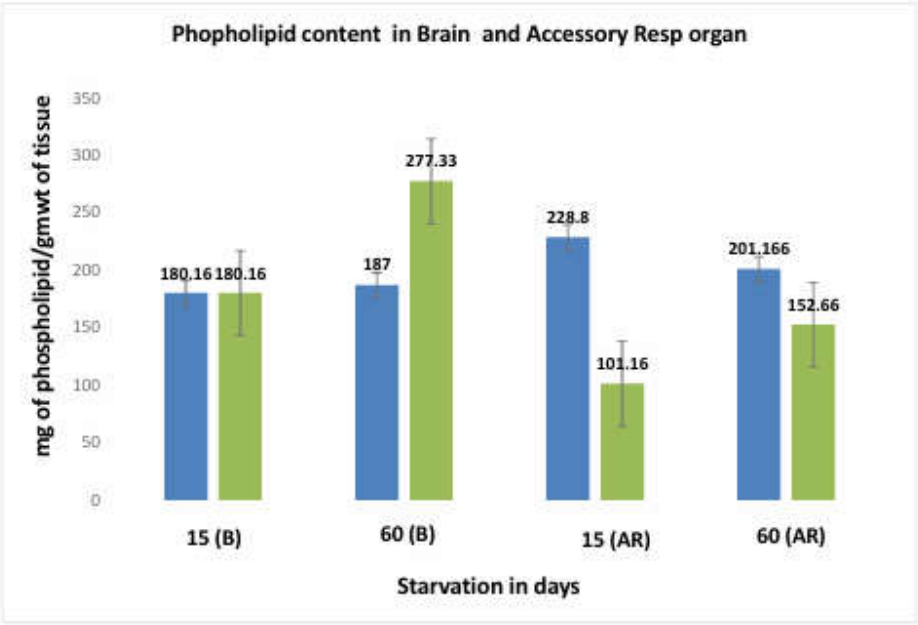

Figure 2: Phopholipid content in Brain and Accessory Resp organ

Table 3: Phospholipid Levels in Pectoral and Lateral Line Muscles during Short Term and Long Term starvation in *A.testudineus*

S. No.	Tissue analyzed	Short Term (15 Days)		Long Term (60 Days)	
		Control	Expt.	Control	Expt.
1.	Pectoral Muscle	165 SE = ± 8.8	93* SE = ± 8.4	188.333 SE = ± 11.1	152.333^{NS} SE = ± 26.6
		% Variation = -43.636		% Variation = -23.684	
2.	Lateral Line Muscle	218 SE = ± 9.3	139* SE = ± 10.1	254.5 SE = ± 10.2	147.5* SE = ± 16.6
		% Variation = -36.238		% Variation = -42.04	

Values expressed in mg of phospholipid / gm wt. of tissue
Each value is mean of SE ± of 6 individual observations
P < 0.01*, NS = Not Significant

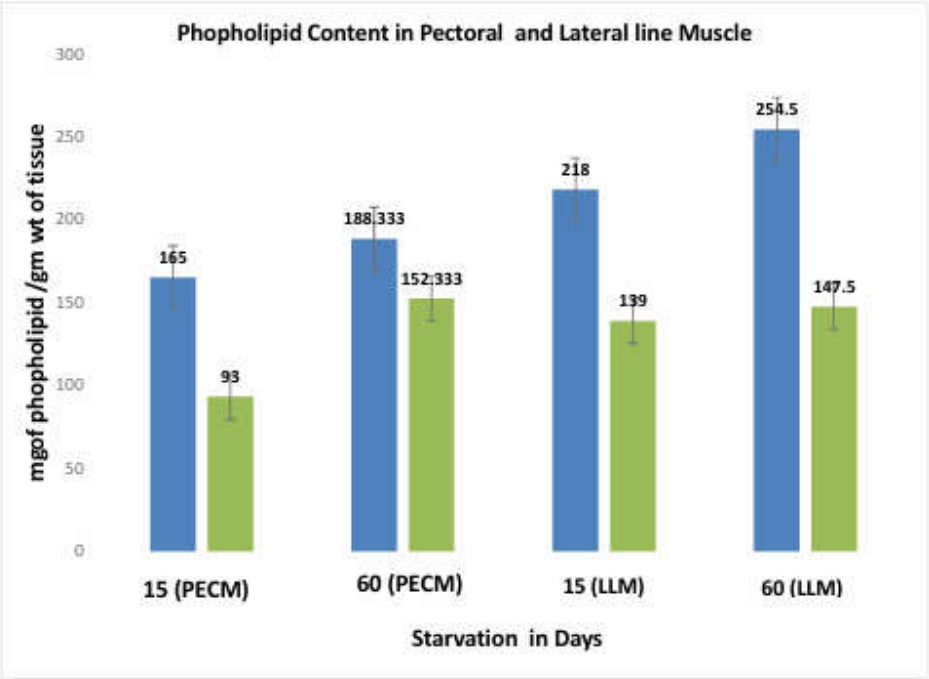

Figure 3: Phopholipid Content in Pectoral and Lateral line Muscle

As there was a differential utilization of phospholipids in the present study, to know the phospholipid utilization over a period of time, (t=0 days to t=60 days) represented as area under the curve (AUC) was calculated using integral calculus.

Liver tissue

The Control Group Integral Setup, for the control group, we have the phospholipid concentration function:

P control (t) =5.44·t+435.4

To find the cumulative phospholipid levels from day 0 to day 60, we set up an integral from t=0, t=60:

$$\int_0^{60} \text{Pcontrol}(t)dt = \int_0^{60} (5.44 \cdot t + 435.4)dt$$

- t is the variable representing time in days.
- dt t dt represents a tiny change in time, or an infinitesimally small interval along the time axis.

$$\int_0^{60} (5.44 \cdot t + 435.4)dt = [2.72 \cdot t2 + 435.4 \cdot t \int_0^{60}$$

Substituting t=60 in to the expression then $2.72.t^2 + 435.4.t$ for t-60

$2.72.60^2 + 435.4.60 = 2.72.3600 + 435.4.60 = 9792 + 26124 = 35916$mg

For t=0

$2.72.0^2 + 435.4.0 = 0$

So integral from 0 to 60 it is 35916-0=35916mg/ gm wt of tissue. So it may be said that 35916 mg of phospholipid was accumulated over a period of time i.e. from 0 days to 60 days. The value 35,916 mg· represents the total phospholipid accumulation in the liver tissue over the 60-day period for the control group. This gives a cumulative measure, telling us how much phospholipid was present in total across the entire period.

For the experimental group, the phospholipid concentrations were given as follows:

- At t=15 days, phospholipid level was 1362 mg/gmwt.
- At t=60 days, phospholipid level was 1425mg/gmwt.
- These data points allow us to create a linear equation to represent the phospholipid concentration over time, in the form

P experimental(t) =m·t+c
1362=1.4·15+c, 1362=21+c, 1362 = 21 + c, 1362=21+c
C=1362-21=1341

$$\int_0^{60} Pexpt\ t.dt = \int_0^{60} (1.4.t + 1341)dt$$

Evaluating the Integral from t=0 to t =60
Now we substitute t= 60 and t=0 into the expression
For t=60
0.7. 60^2 + 1341.60 = 0.7.3600+ 1341.60= 2520+ 80460=82980
For t =0
0.7·02+1341·0=0
So, the result of the integral from t= 0 to t=60
82980−0 = 82980 mg/gm wt. of tissue.

The value **82,980 mg** represents the **total phospholipid accumulation** in the liver tissue over the 60-day period for the experimental group. This tells us how much phospholipid was present in total over this period, giving a cumulative measure for the experimental conditions.

In conclusion, the integration of both control and experimental phospholipid levels over 60 days shows a significant increase in total phospholipid accumulation in the experimental group, likely due to the impact of the specific conditions applied in this experiment. This cumulative analysis highlights how the experimental condition might be promoting a sustained higher level of phospholipid production or retention over time.

Similarly for all the tissues, the cumulative phospholipid value was calculated using integration and the results are as follows.

Kidney: Cumulative phospholipid over the time, from t=0 to t=60 is as follows.
Control group: 14268.67mg **Experimental (starvation) group:** 19140.00mg
The experimental group shows a cumulative increase of **4,871.33 mg**· in phospholipid levels compared to the control group over the 60-day period. This suggests that the experimental conditions (e.g., prolonged starvation) resulted in a **higher retention or synthesis** of phospholipids in kidney tissue over time.

Brain: Cumulative phospholipid over the time, from t=0 to t=60 is as follows.
Control Group: 10,946.4 mg· **Experimental Group**: 12,752.7 mg·
The experimental group shows a cumulative increase of **1,806.3 mg**· in phospholipid levels compared to the control group over the 60-day period.

Accessory Respiratory Organ
Cumulative phospholipid over the time, from t=0 to t=60 is as follows.
Control Group: 13,185.3 mg· **Experimental Group**: 7,117.23 mg·
The control group shows a significantly higher cumulative phospholipid level (**6,068.07 mg· more**) than the experimental group over the 60-day period. This suggests that, under experimental conditions (prolonged starvation), the accessory respiratory organ utilized or lost phospholipids at a much higher rate, resulting in a significantly lower accumulation compared to the control group.

Pectoral Muscle
Cumulative phospholipid over the time, from t=0 to t=60 is as follows.
Control Group: 10,366.56 mg·; **Experimental Group**: 6,766.56 mg
The control group shows a cumulative increase of **3,600 mg**· in phospholipid levels compared to the experimental group over the 60-day period. This suggests that, under experimental conditions (starvation), the pectoral muscle utilized or lost phospholipids at a higher rate, resulting in significantly lower cumulative levels compared to the control group.

Lateral Line Muscle
Cumulative phospholipid over the time, from t=0 to t=60 is as follows.
Control Group: 13,809.99 mg·; **Experimental Group**: 8,510.01 mg
The control group shows a significantly higher cumulative phospholipid level (**5,299.98 mg· more**) compared to the experimental group over the 60-day period. This indicates that, under starvation conditions, the lateral line muscle of fish utilized or lost phospholipids more rapidly, resulting in a lower overall accumulation compared to the control group.

DISCUSSION

In the present investigation on *anabas,* revealed that it is has adapted well for the new stressful situation of food deprivation. Though the metabolic activity seemed to be depressed and the fish movements were slowed down, no mortality was reported during the starvation period. This clearly indicates that *Anabas testudineus*, has successfully adapted to the stressful situation by making certain physiological adjustments. Short-term fasting (15 days) and prolonged fasting (60 days) yielded mixed results, which are summarized as follows. Both terms of starvation resulted, in a significant rise of phospholipids in tissues such as liver and kidney. Brain, also showed an increase only during the long term starvation; however during short term fasting there was no change observed. In sharp contrast to these results, tissues such as accessory respiratory organ, pectoral and lateral line muscle showed a considerable decline, in both the starvation regimes, probably suggesting that they may have been utilized as energy substrates, by the starving animal. The rise of phospholipids found in liver and kidney coincides with the observations of Love (1980), and Dave *et.al,* 1975) who have observed a similar kind of upheaval of phospholipids, during starvation in *Gadus moruha* and *Anguilla anguilla* respectively. This rise of phospholipids may be due to a simultaneous synthesis occurring along the degradation, as a certain threshold level of phospholipids must be maintained, all throughout so as to stabilize the structural integrity of cellular membranes, as this becomes, quite necessary to overcome the stressful starvation and thereby enhance the survival chances of the fish.(Kanazawa *et.al,* 1985).Apart from being identified as passive structural entities, contributing to the compactness and integrity of the membranes, recent evidences suggest that they are identified as active biological signaling molecules, as endogenous ligands, for the development of adipose tissue and lipid metabolism and also involved in differentiation of other cell types.(Dean and Lodhi, 2017).Therefore the elevation in certain tissues may be attributed to a concurrent synthesis, occurring along the degradation probably to achieve any of the said functions. The decline in phospholipids, observed in accessory respiratory organ, pectoral and lateral line muscle, of *Anabas*, are in accordance with the observations made by Ross (1978), Johnston and Pink (1973c) Deual (1951) and Anasell and Hawthone (1964) who have all also reported a similar decline in structural lipids (Cholesterol and Phospholipids) in muscle during starvation, suggesting that phospholipids may be degraded to satisfy the energy demands of the starving fish**.** These results coincide with the observations of Wu, Xugan *et.al*, (2017) who have worked on starvation on crabs belonging to the species**,** *Portunus pelagicus.* In their work, they had reported that phospholipids, are the major lipid classes which were utilized during starvation, especially in newly hatched larvae and had observed a 50 % decline in phospholipid concentration. In another study by Kevin P Kelly *et.al*, (2022) who have studied phospholipids and enzymes related to its metabolism in *Drosophila,* suggest that phospholipid homeostasis is essential for insulin sensitivity and hunger response. Based upon, these results it may be said that, during starvation, phospholipid homeostasis may be crucial, for membrane stability and also to maintain insulin sensitivity. Therefore the augmented phospholipid concentration in certain tissues during both terms of starvation may be attributed to their simultaneous synthesis, occurring along degradation so as to maintain homeostasis, which may be crucial to overcome the starvation stress. In sum it may be said, that when *anabas,* when subjected to brief and prolonged fasting, the animal adapted, successfully by deriving energy from the lipid source, such as phospholipids, and at the same time, strives, to maintain, the integrity of membranes, so as to stabilize the structural dynamics of the cell, by synthesizing them, in certain vital tissues, which becomes, compelling to the starving animal, to beat the stress and there by surviving all through the starvation period.

CONCLUSION

From the above results, we conclude that the phospholipids as hypothesized seem to be the key players as energy substrates and also as

successful integrators and stabilizers aiding in packaging and tightening the membranes during starvation induced stress there by assisting the animal to overcome the adversities and enhance the chances of survival.

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