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# Dynamics of immune enzymes in the haemolymph of different breeds of silkworm *Bombyx mori* L during the progress of fungal pathogen *Beauveria bassiana* (Bals.) Vuill.

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**Abstract**

Dynamics of immune enzymes viz., catalase and phenoloxidase was investigated in the haemolymph of 5th instar silkworm ***Bombyx mori*** from 1st to 6th day in all three breeds viz., crossbreed, bivoltine single hybrid and bivoltine double hybrid chosen for the study in ***Beauveria bassiana*** inoculated silkworms. Reduction of catalase enzyme activity was observed in haemolymph of both inoculated and control batches from 1st to 6th day of the 5th instar silkworms. However, significant reduction of catalase enzyme activity was recorded in ***Beauveria bassiana*** inoculated larvae in contrast to control. Gradual increase of the phenoloxidase enzyme activity was recorded up to 4th day in bivoltine double hybrid and 3rd day in both crossbreed and bivoltine single hybrid then the significant reduction of phenoloxidase was noticed in the rest of the instar in ***Beauveria bassiana*** treated silkworms compared to control. Maximum level of Catalase and Phenoloxidase activity was observed in double hybrids followed by crossbreed and single hybrid. The research investigation very distinctly indicated the catalase and phenoloxidase enzyme dynamics in the blood of silkworm ***Bombyx mori*** and its association with silkworm resistance against fungal pathogen ***Beauveria bassiana.***

Keywords: ***Bombyx mori***, ***Beauveria bassiana***, Haemolymph, catalase, Phenoloxidase

## Introduction

Sericulture is the art of culturing of silkworms for the production of silk and it is an agro-based cottage industry providing gainful employment. The prime activities of sericulture are cultivation of mulberry the sole food for silkworm ***Bombyx mori,*** cocoon production the basic raw material for silk industry, silk reeling, twisting and weaving. Due to continuous domestication for centuries silkworms become very sensitive and prone to attack of number diseases caused by different pathogenic micro-organisms viz., protozoan, viral, bacterial and fungal pathogens. White muscardine is the most common fungal disease in silkworm caused

by ***Beauveria bassiana*** and it was the first microorganismreported in insects. The fungal pathogen is most virulent and the disease develops very rapidly during rainy and winter seasons. Sudhakara Rao *et al* (2011) estimated that 27 to 35 percent of cocoon crop loss is due to silkworm diseases and the yield of cocoon crop reduces by 11-15 kg/100 DFLs. In total cocoon crop loss 10 to 40 percent of loss has been accounted for white muscardine disease caused by ***Beauveria bassiana*** in India (Chandrasekharan and Nataraju 2008). The mode of fungal infection is per cutaneous and the conidia grow and enter through the integument and establish infection in silkworm ***Bombyx mori.*** The germ tube enters into the host and proliferates into hypodermal region then enters into haemocoel and it remains in the blood and draws the nourishment from haemolymph and disrupts the haemolymph and fat body cells prior to death of the host insect. Haemolymph is a lifesaving complex circulating tissue fluid that maintains the appropriate environment in the host system. It is composed of plasma and haemocytes and plays very significant role in the defense mechanism and transportation of vital inorganic and organic biomolecules and nutrients throughout the body and haemocytes are an important constituent of the insect defense mechanism.

Strand (2008) suggested that cell-mediated and humoral responses are major components of innate immune mechanism in insects. Lavine and Strand (2002)and strand (2008) mentioned that cellular immune responses comprises of phagocytosis of tiny foreign particles that enters into the insect body, encapsulation of large pathogens or parasites and nodule formation are most essential immune responses in insects by haemocytes. Humoral immune response is mediated by humoral antibacterial peptides (AMP) generated via immune deficiency (Imd) pathways and numerous immune proteins (Lemaitre and Hoffmann, 2007).The same researchers reported that the melanization process was induced around the foreign particles by the activated humoral protein viz., Pro-phenloxidase and at the same time it persuades cellular and humoral immunity in the host system. Zhao *et al* (2007) reported that intermediates that produced in the process of melanisation were capable to terminate bacterial pathogens directly. In insects the innate defence mechanism is comprised of wide range of specific and non- specific reactions that are actuated in the presence of foreign agents. One of the essentialfactors in such responses is the enzyme phenoloxidase. Phenoloxidase enzyme plays an important role in identification of microorganisms and fight against the pathogenic microorganism in invertebrates and it is a terminal enzyme in the process of melanisation. Catalase is an essential antioxidantenzyme

present in almost all animals. The enzyme catalyzes the degradation of hydrogen peroxide to water and oxygen and protects the organism from oxidative damage. With this backdrop the researchers made an attempt to examine the dynamics of the immune enzymes i.e., catalase and phenoloxidase inoculated with fungal pathogen ***Beauveria bassiana*** in silkworm ***Bombyx mori***.

## Materials and Methods

***Beauveria bassiana*** pure culture was maintained under aseptic condition in the laboratory. Immediately after fourth moult, the silkworms were treated with ***Beauveria bassiana*** spore suspension in sub-lethal concentration (2.15 X $10^6$ conidia/ml) in crossbreed and bivoltine single hybrid and 2.15 X $10^4$ conidia/ml in bivoltine double hybrid) for 45 seconds. For control the healthy silkworms were treated with double distilled water. Haemolymph of the $5^{th}$ instar silkworms treated with fungal spore suspension was collected after 24 hours of treatment to examine the day to day changes in catalase and phenoloxidase enzyme activity. The haemolymph was collected from the anesthetized silkworms at low temperature after surface sterilization with 70 percent of ethyl alcohol.By using sterile blade small incision was made on each abdominal leg and haemolymph was collected in sterile eppendof tubes from the three breeds of silkworms viz., PM x CSR2 (crossbreed), CSR2 × CSR4 (bivoltine hybrid), and (CSR2 X CSR27) X (CSR6 X CSR 26) (bivoltine double hybrid) selected for the study. A pinch of phenylthiourea was added to avoid the melanization in the haemolymph samples and stored at $20^oC$. The enzyme activity of catalase (Aebi, 1974) and phenoloxidase (Mason, 1947) was measured by usingstandard protocols. Four replicates were maintained with hundred silkworms in each replication for the experiment.

## Results and Discussion

The prevalence of the white muscardine disease caused by ***Beauveria bassiana*** is very high during rainy and winter seasons, as reported by previous workers Janakiram (1961) Chandrasekaran and Nataraju (2005).According to Chandrasekarn and Nataraju (2005) susceptibility of muscardine disease varies among different silkworm breedsnaturally the tolerant breed has immunity against ***Beauveria bassiana*** infection. Mulberry silkworm is a tiny insect with a lot of commercial importance and is the best promising laboratory tool to carryout research studies on insect defense mechanism that haswell-organized innate immune system against aggressive pathogenic microorganisms.Therefore in depth investigations on

silkworm humoral innate immunity is usefulfor hypothetical and practical applications. Hence an attempt has been made to determine the catalase and phenoloxidase enzyme activity in haemolymph of three silkwormbreeds viz., PM x CSR2 (crossbreed), CSR2 × CSR4 (bivoltine hybrid), and (CSR2 X CSR27) X (CSR6 X CSR 26) (bivoltine double hybrid) during the progress of fungal pathogen ***Beauveria bassiana***.

The results on catalase enzyme activity are presented in table-1 and graph-1. Gradual decline of catalase activity was noticed in untreated silkworms during 5$^{th}$instari.e., from 1$^{st}$ to 6$^{th}$ day in double hybrid(4.83 µ moles/mg protein/min to 2.27 µ moles/mg protein/min) followed by crossbreed (3.95 µ moles/mg protein/min to 1.94 µ moles/mg protein/min) and single hybrid (3.48 µ moles/mg protein/min to 1.81 µ moles/mg protein/min).In ***Beauveria bassiana*** infected larvae, significant reduction of the catalase activity was noticed throughout the 5$^{th}$ instar in all three breeds i.e., double hybrids(4.41µ moles/mg protein/min to 1.87µ moles/mg protein/min) followed by crossbreed (3.5 µ moles/mg protein/min to 1.36 µ moles/mg protein/min) and in single hybrid (3.21 µ moles/mg protein/min to 1.24 µ moles/mg protein/min) compared to healthy silkworms. Double hybrid showed higher level of catalase enzyme activity throughout the 5$^{th}$ instar silkworm both in inoculated and control compared to crossbreed and single hybrid.

Catalase enzyme plays a key role in host defense mechanism that is prerequisite during host-pathogen interaction in the host system for survival. It is a vital antioxidant enzyme that reduces the oxidative stress and protects the cell from oxidative damage (Bandopadhyay et al 1999). Hao *et al* (2003) reported that Reactive Oxygen Species (ROS) plays a vital role in non-specific defense mechanism. Kumar *et al* (2003) reported that catalase enzyme stimulates signal transduction and facilitates several biochemical reactions such as cell growth and apoptosis.Elevation of ROS levels due to stress induced by the pathogen may results disruption of cell structures and regular mechanisms of cellular signalling. Cumulatively, it is known as oxidative stress. Catalase has highest rate of catalytic activity; in each second one moleculeof catalase can breakdown millions of hydrogen peroxide molecules to water and oxygen. Regular metabolic activities generate injurious by-product namely Hydrogen peroxide (H2O2)it should be immediately breakdown to oxygen and water otherwise it damages cells and tissues and also damagevital biomolecules such as DNA, protein and lipids.

Felton and Summers (1995) emphasized the importance of catalase enzyme in defense responses in insects. Wu Xiaofeng and Xu Junliang (1998) reported the catalase enzyme activity in the blood of silkworm ***Bombyx mori*** and its relationship to disease resistance in silkworm. Switala and Loewen (2002) reported that catalase enzyme, reduces the Hydrogen peroxide (H2O2) to water and oxygen. Fornazier *et al* (2002) opined that thecatalase enzymeactivity was enoughto deal with high concentration of hydrogen peroxide treatedwith Cd2+ and the cells expressed a positive endogenic protective effect.Alvaro Molina-Cruz*et al* (2007) reported that suppression of midgut catalase in ***Anopheles gambiae*** lead to higher levels of hydrogen peroxide against bacteria and ***Plasmodium***and that leads to mortality. Madhusudhan *et al* (2012) observed the reduction of the catalase enzyme activity in pebrine inoculated tasar silkworm and indicated that catalase activity can be used as anindex to measure the health status of ***Antheraea mylitta*** Drury. Wang *et al* (2013) stated that prevention of oxidative stressis beneficial to appropriateimmune responses in immune cells. Nicolás Pedrini *et al (*2013) reported that peroxisomal catalases might be vital elements for adaptation to oxidative stress produced during the development of fungal pathogen *Beauveria bassiana.*

Significant reduction of catalase activity was recorded in three hybrids selected viz., bivoltine double hybrid, crossbreed and bivoltine single hybrid for the investigation. High level of elevation of catalase enzyme activity was noticed in bivoltine double hybrid followed by crossbreed and bivoltine single hybrid. Depletion of catalase enzyme activity in ***Beauveria bassiana*** treated silkworm was attributed to the possible damage to peroxisomes in the cell that may lead to accumulation of H2O2 in the ***Beauveria bassiana*** inoculated silkworms, which may cause the mortality in the treated silkworms as H2O2 is cytotoxic. Another probable reason for depletion of catalase enzyme activity in infested silkworm may be due to low ingestion of food and metabolic modifications as a part of the defense mechanism due to invasion of fungal pathogen. Elevation of antioxidant enzyme catalase activity in bivoltine double hybrid indicates higher level of immunity compared to other two breeds selected for the study. Enhanced antioxidant enzyme activity may support the defense mechanism of host organism Significant reduction of catalase activity was recorded in three hybrids selected viz., bivoltine double hybrid, crossbreed and bivoltine single hybrid for the investigation. High level of elevation of catalase enzyme activity was noticed in bivoltine double hybrid followed by crossbreed and bivoltine single hybrid. Depletion of catalase enzyme activity in ***Beauveria bassiana*** treated silkworm was attributed to the possible damage to peroxisomes

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Day to Day alterations in phenoloxidase activity was examined in three breeds selected for the study during the development of fungal pathogen ***Beauveria bassiana*** and the data is presented in Table-2 and Graph-2. In untreated silkworms reduction of phenoloxidase activity was noticed from $1^{st}$ to $6^{th}$ day in all three breeds selected for the study i.e., double hybrid (0.56μ moles/mg protein/mint 0.25 μ moles/mg protein/min), crossbreed (0.45 μ moles/mg protein/mint 0.2 μ moles/mg protein/min) and single hybrid (0.4μ moles/mg protein/min to 0.19 μ moles/mg protein/min). In double hybrid, a gradual increase of the phenoloxidase was recorded up to $4^{th}$ day (0.51 μ moles/mg protein/ min to 0.57μ moles/mg protein/min) and then the significant reduction of phenoloxidase was noticed (0.33 μ moles/mg protein/min to 0.28 μ moles/mg protein/min) in rest of the instar of treated larvae.

In case of crossbreed and single hybrid gradual elevation of phenoloxidase activity was recorded up to $3^{rd}$ day (0.44 μ moles/mg protein/min to 0.49 μ moles/mg protein/min) and (0.43 μ moles/mg protein/min to 0.46 μ moles/mg protein/min) respectively then substantial reduction of phenoloxidase enzyme activity was recorded in the remaining days of the instar in crossbreed (0.32 μ moles/mg protein/min to 0.14 μ moles/mg protein/min) and in single hybrid (0.27 μ moles/mg protein/min to 0.12 μ moles/mg protein/min) with reference to untreated silkworms. Maximum level of phenoloxidase enzyme activity was noticed in bivoltine double hybrids followed by crossbreed and bivoltine single hybrid. Pattern recognition receptors activate downstream serine protease cascades that terminate the activation of prophenoloxidase, prior to prophenoloxidase minimum two proteases are activated that are present in the blood of insect all the time as an inactive proenzyme and it is converted to the active form immediately after the ingress of a foreign agents into the insect system. Active phenoloxidase is sticky to the foreign particles encapsulated and darkened

where it aids localized melanization. Melanisation is a response triggered by phenoloxidase in insects by the entry of pathogenic organisms and plays significant role in various biological activities that includes wound healing, tanning of the cuticle and immunity. The quinones produced during the action of phenoloxidase are either used for melanin formation or destroyed by quinone isomerase. The later operation stops the dissemination of quinones to other cellular sites and any possible damage of self-matter. Protease inhibitors regulate the undesired activation of the prophenoloxidase cascade.

Insects have a broad spectrum of immune mechanism to fight against the invading pathogens/parasites/foreign particles even though they do not have complicated immunoglobulins. Brookman *et al* (1988), Nappi and Christensen (1987) and Boucias and Latge (1988) opined that phenoloxidase system is a simple mechanism that exists in immune and non-immune insects and involved in the process of phagocytosis, production of melanin for wound healing and to build humoral and cellular capsules. Sugumaran and Kanost (1993) reported that prophenoloxidase cascade is a major factor in insect immune mechanism. Elevation of phenoloxidase enzyme activity was noticed in the initial stages of ***Beauveria bassiana*** infection. The enhancement of the phenoloxidase enzyme activity may be due to invasion of ***Beauveria bassiana*** into the host blood as phenoloxidase was present in haemolymph and it turns into active form that results in enhancement of phenoloxidase during infection and quinines are also released by the host system in the process of melanisation that may be lethal to the microorganisms invaded into the host system. Decreased level of phenoloxidase enzyme activity was noticed in the advanced stage of infection it might be owing to suppression of the enzyme activity by inhibitor factors secreted by the fungal pathogen ***Beauveria bassiana*** and also due to cessation of feed by the host insect in advanced stage of infection. Since the production and maintenance cost of the phenoloxidase and prophenoloxidase activating system is high because tyrosine is the chief compound for prophenoloxidase activating system obtained from phenylalanine that can only be synthesized from the food ingested (Chapman 1998).

Cerenius and So¨derha¨ll (2004) reported that prophenoloxidase synthesis occurs mostly in haemocytes with species-specific variation in relation to haemocytes type. Jiang *et al* (1997) stated that the site of prophenoloxidase (proPO) synthesis is oenocytoids, a type of haemocytes in lepidopteron insects. Iwama and Ashida (1986) stated that oenocytoids synthesize the prophenoloxidase enzyme and released into plasma. Tang (2006) reported that

quinines are the products of phenoloxidase responses that may be lethal to pathogenic microorganisms. Ashida (1971 and 1983) and Ashida and Brey (1998) isolated and purified two components viz., PPO and β-1,3-glucan binding protein of the PPO system in silkworm ***Bombyx mori***. Dohke (1973) isolated a serine proteinase from the silkworm ***Bombyx mori*** cuticle that can activate prophenoloxidase and also stated that plasma of silkworm haemolymph have pro-phenoloxidase and the activating system for the pro-enzyme.

## Conclusion

It is reported that pathogenic micro-organisms induces a number of biochemical and physiological anomalies in the insect host system. Metabolic anomalies and fluctuations in the enzymatic activity during the development of pathogenic organism in the host system play a significant role in understanding the host and pathogen interactions to fight against the stress induced by the pathogen. Energy is essential for all living organisms for the growth and development; enzymes are the major source of energy for organisms for the sustainable metabolic activities. In an organism each and every function has its specific enzyme to perform the definite task. Rajitha and Savithri (2014) reported that catalase is a very important enzyme that breakdown the hydrogen peroxide (H2O2) into water and oxygen and protect the cell from oxidative stress caused by reactive oxygen species (ROS).Phenoloxidase is an immune enzyme that plays a crucial role in recognition of foreign particles and fight against pathogenic micro-organisms in invertebrates. The current investigation primarily concentrated to understand the degree of defence responses for microbial infection in three silkworm breeds chosen for the study using catalase and phenoloxidase enzyme activity as an index. Among the three breeds chosen for the research investigation, bivoltine double hybrid showed higher level of enzyme activity that indicates higher degree of tolerancefollowed by crossbreed and single hybrid against fungal pathogen ***Beauveria bassiana.*** Dynamics of catalase and phenoloxidase enzyme activity could be used as a directory to segregate the silkworm breeds for disease resistance.

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**Table 1: Dynamics of catalase activity (µ moles/mg protein/min) in the haemolymph of silkworm *Bombyx mori* L. treated with fungal pathogen *Beauveria bassiana* (Bals.) Vuill. in three breeds selected for the study during fifth instar compared to control.**

| CATALASE | | | | |
|---|---|---|---|---|
| S.NO | Days | (CSR 2 x CSR 27) x (CSR 6 x CSR 26) | (PM X CSR2) | (CSR2 X CSR 4) |

| | | Control | Inoculated | Control | Inoculated | Control | Inoculated |
|---|---|---|---|---|---|---|---|
| 1 | Day 1 | 4.83 | 4.41 | 3.95 | 3.5 | 3.48 | 3.21 |
| 2 | Day 2 | 4.43 | 3.34 | 3.78 | 3.12 | 3.31 | 3 |
| 3 | Day 3 | 4.19 | 3.27 | 3.43 | 3.08 | 3.26 | 2.55 |
| 4 | Day 4 | 3.95 | 3.14 | 3.18 | 2.02 | 3.16 | 1.95 |
| 5 | Day 5 | 3.57 | 2.54 | 2.53 | 1.89 | 2.41 | 1.57 |
| 6 | Day 6 | 2.72 | 1.87 | 1.94 | 1.36 | 1.81 | 1.24 |
| Mean | | 0.464 | 3.095 | 3.135 | 2.648 | 2.907 | 2.253 |
| Std. Deviation | | 0.0994 | 0.8017 | 0.725 | 1.218 | 0.616 | 0.822 |

Tests of Between-Subjects Effects
Dependent Variable: CATALASE

| Source | Type III Sum of Squares | df | Mean Square | F | P value | SIG |
|---|---|---|---|---|---|---|
| Breeds | 16.580 | 2 | 8.290 | 51.967 | 0.000 | ** |
| Treatments | 11.927 | 1 | 11.927 | 74.766 | 0.000 | ** |
| Days | 53.862 | 5 | 10.772 | 67.530 | 0.000 | ** |
| Breeds*Treatments | 0.609 | 2 | 0.304 | 1.907 | 0.156 | NS |
| Breeds* Days | 2.288 | 10 | 0.229 | 1.434 | 0.183 | NS |
| Treatments * Days | 1.628 | 5 | 0.326 | 2.042 | 0.083 | ** |
| Breeds *Treatments * Days | 2.005 | 10 | 0.200 | 1.257 | 0.271 | NS |
| Error | 11.486 | 72 | 0.160 | | | |
| Corrected Total | 100.384 | 107 | | | | |
| a R Squared = .886 (Adjusted R Squared = .830) | | | | | | |

p<0.05 Significant at 0.05 level p<0.01 significant at 0.01 level p>0.5 Not significant

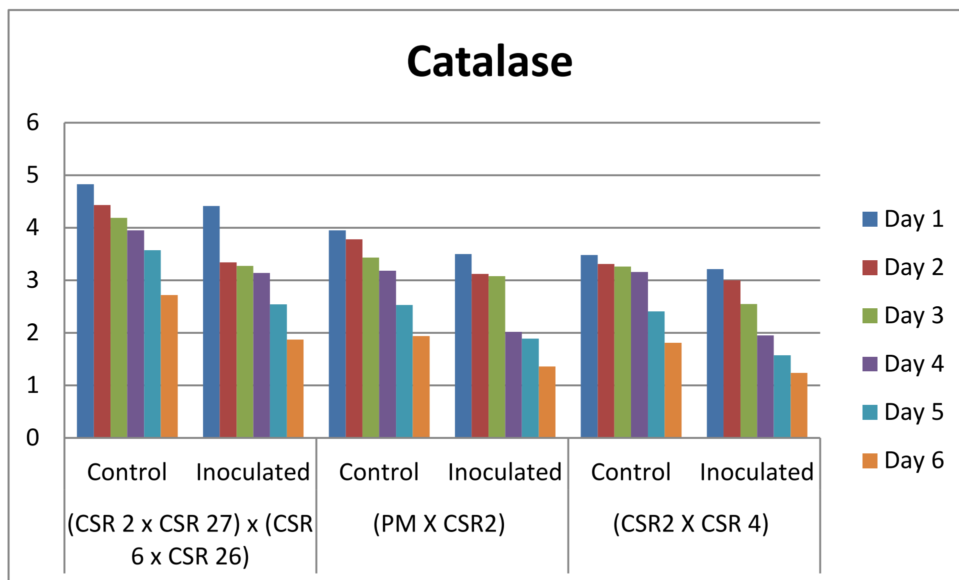

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**Table 2: Dynamics of phenoloxidase activity (µ moles/mg protein/min) in the haemolymph of silkworm *Bombyx mori* L. treated with fungal pathogen *Beauveria bassiana* (Bals.) Vuill. in three breeds selected for the study during fifth instar compared to control.**

| PHENOLOXIDASE | | | | | | | |
|---|---|---|---|---|---|---|---|
| S.NO | Days | (CSR 2 x CSR 27) x (CSR 6 x CSR 26) | | (PM X CSR2) | | (CSR2 X CSR 4) | |
| | | Control | Inoculated | Control | Inoculated | Control | Inoculated |
| 1 | Day 1 | 0.56 | 0.51 | 0.45 | 0.44 | 0.4 | 0.44 |
| 2 | Day 2 | 0.54 | 0.53 | 0.42 | 0.47 | 0.39 | 0.45 |
| 3 | Day 3 | 0.48 | 0.56 | 0.41 | 0.49 | 0.39 | 0.46 |
| 4 | Day 4 | 0.36 | 0.57 | 0.33 | 0.32 | 0.32 | 0.27 |
| 5 | Day 5 | 0.30 | 0.33 | 0.31 | 0.26 | 0.25 | 0.23 |
| 6 | Day 6 | 0.25 | 0.28 | 0.2 | 0.14 | 0.19 | 0.12 |
| Mean | | 0.4645 | 0.467 | 0.356 | 0.357 | 0.326 | 0.328 |
| Std. Deviation | | 0.099 | 0.118 | 0.11 | 0.1362 | 0.0847 | 0.137 |

| Tests of Between-Subjects Effects<br>Dependent Variable: PHENOLOXIDASE | | | | | | |
|---|---|---|---|---|---|---|
| Source | Type III Sum of Squares | df | Mean Square | F | P value | SIG |
| Breeds | 0.369 | 2 | 0.185 | 109.667 | 0.000 | ** |
| Treatments | 0.000 | 1 | 0.000 | 0.205 | 0.652 | NS |
| Days | 1.174 | 5 | 0.235 | 139.461 | 0.000 | ** |
| Breeds*Treatments | 0.000 | 2 | 0.000 | 0.056 | 0.945 | NS |
| Breeds* Days | 0.027 | 10 | 0.003 | 1.625 | 0.117 | NS |
| Treatments * Days | 0.036 | 5 | 0.007 | 4.333 | 0.002 | ** |
| Breeds *Treatments * Days | 0.016 | 10 | 0.002 | 0.948 | 0.496 | NS |
| Error | 0.121 | 72 | 0.002 | | | |
| Corrected Total | 1.744 | 107 | | | | |
| a R Squared = .931 (Adjusted R Squared = .897) | | | | | | |

p<0.05 Significant at 0.05 level p<0.01 significant at 0.01 level p>0.5 Not significant