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Research Article

Validity of Visible Ectoparasite Intensity As a Non-invasive Biomarker for Fish welfare: Parasitic Copepod, *Lernantropus kroyeri* in Sea Bass As an Example

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ABSTRACT

Ensuring fish welfare is essential from the ethical, legal, environmental, economic, and social perspectives. It plays a vital role in maintaining the health and sustainability of aquaculture practices while respecting the intrinsic value and welfare of the fish themselves. The presence of reliable welfare assessment schemes is of utmost importance to appraise the well-being of animals in aquaculture and uphold stringent welfare standards. In determining fish welfare, conducting welfare assessments with non-invasive biomarkers is crucial thus the primary objective of this study is to explore the potential usability of visible parasites as welfare biomarkers in fish without causing any harm to the fish. In this research, certain secondary stress indicators (hematocrit, plasma glucose and lactate) were employed as biomarkers for assessing the well-being of European sea bass (*Dicentrarchus labrax*). The study aimed to investigate whether there is a possible correlation between the presence of visible ectoparasites on the gills (specifically, the Copepod parasite, *Lernantropus kroyeri*) and the aforementioned stress parameters. Thus, in this study, the examination was conducted to establish the validity of ectoparasites as non-invasive biomarkers for evaluating the welfare of fish. The results showed that there was a statistically significant relationship between the intensity of ectoparasites and the stress parameters used as indicators of welfare. The observable presence and intensity of ectoparasites on the gills of the fish can be proposed as a non-invasive biomarker for evaluating fish welfare in aquaculture.

Keywords: Fish welfare, non-invasive biomarker, ectoparasite intensity, validity

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INTRODUCTION

The importance of animal welfare in the aquaculture industry, which is responsible for producing 52% of the fish consumed by humans (FAO, 2022) has started to be better understood. Poor welfare conditions in aquaculture can lead to negative outcomes for the environment, animal well-being, and global food security. By promoting fish well-being, the aquaculture industry can contribute to improving sustainability and fish food safety, which is beneficial for the environment and human health. Therefore, it is crucial to ensure sustainable growth in aquaculture while prioritizing fish welfare.

Non-invasive biomarkers for assessing fish welfare have gained significant attention in recent years. These biomarkers allow for the evaluation of fish well-being without causing unnecessary stress or harm to the animals. The need for non-invasive fish welfare biomarkers arises from the increasing concern and recognition of the ethical and practical importance of fish welfare in various industries, including aquaculture, fisheries, and research. Fish are sentient beings capable of experiencing pain, stress, and suffering, and as such, there is a growing responsibility to ensure their well-being and minimize any potential harm they may experience (Garrath & McCulloch, 2022). Assessing fish welfare has been challenging due to their environment and the fact that they are not as expressive as some land animals. Many conventional welfare assessment methods involve capturing, handling, or even sacrificing fish for analysis, which can cause additional stress and harm to the animals (Browning, 2023). Non-invasive biomarkers offer a more humane and sustainable approach to monitoring and assessing fish welfare. These biomarkers can be indicators of physiological, biochemical, or molecular changes in fish, providing valuable insights into their well-being without causing harm or stress. For example, cortisol concentration in fish skin mucus has been proposed as a non-invasive technique to assess fish welfare and stress (Guardiala et al., 2016; Carbajal et al., 2019). Similarly, the cutaneous stress response system (CSRS) in fish skin has been reported as a new source of information on the welfare status of farmed fish (Kulcykowska, 2019). Although not strictly biomarkers, observing fish behavior can provide valuable insights into their welfare. Non-invasive methods such as video recording or automated image analysis can be used to monitor behaviors such as feeding patterns, swimming activity, aggression, or abnormal behaviors, which can indicate stress or welfare issues (Martins et al., 2012). The establishment of standardized indicators and assessment of welfare needs in aquaculture has been an ongoing discussion (Segner et al., 2019; Magalhães et al., 2020; Barreto et al., 2021; Yavuzcan Yildiz et al., 2021). The use of welfare indicators provides crucial insights into fish welfare, reflecting the extent to which their needs are met. These indicators should possess characteristics such as usability, reliability, scalability, recognizability, minimal damage, and feasibility within a reasonable timeframe. An ideal assessment framework should encompass both operational and laboratory-based welfare markers (Segner et al., 2019; Browning, 2023).

Fish health assessments are one component of on-farm welfare appraisals, particularly in intensive aquaculture systems. Segner et al. (2012) explained that good welfare is reflected in the ability of the fish to cope with various stressors, thereby maintaining homeostasis associated with good health, while stressful aquaculture conditions will cause the loss of coping capacity and impaired health. The welfare status of fish, as measured by the indices of stress can be unveiled. The biochemical and physiological changes result from the effects of the factors released during the primary stress response (Ellis et al., 2012; Schreck & Tort, 2016). The secondary stress response refers to the physiological and behavioral adaptations that occur in response to stressful conditions. These adaptations involve the activation of various metabolic pathways, leading to significant changes in blood chemistry, hematology, respiration, acid-base balance, and ion losses in the gills (Iwama, 2007). Hematocrit, blood lactate, and glucose are considered secondary stress biomarkers in fish. These biomarkers are used to assess the physiological responses of fish to stress and provide valuable information about their overall well-being and health (Seibel et al., 2021).

It is generally recognized that parasitic diseases in fish are one of the most important indicators in relation to fish health (Segner et al., 2012; Stien et al., 2013; Bui et al., 2019). The visible parasites are considered in operational indicators and assessment of fish welfare (Bui et al., 2019), and visible ectoparasites can provide insights into fish well-being without necessarily causing the death of the fish. Thus, the study by Overli (2014) has contributed to our understanding of the relationship between parasites, stress, and welfare. This study revealed that infestation by sea lice led to increased brain stem levels of the 5-Hydroxytryptamine(5-HT) catabolite 5-Hydroxyindolaceticacid (5-HIAA) in Atlantic salmon, indicating a general stress response in infested fish. It was also found that infected fish showed depressed feeding and reduced locomotion, suggesting a negative impact on animal welfare.

The European sea bass (*D. labrax*), one of the most commonly farmed fish in the Mediterranean, faces challenges from the ectoparasite Lernanthropus (Yavuzcan Yildiz & Korkmaz, 2021). *Lernanthropus kroyeri*, belonging to the genus of parasitic copepods, is visible in the gills of sea bass and causes several pathologies such as erosion and necrosis on gill filaments (Henry et al., 2009). The parasite *L. kroyeri* in sea bass has been examined in various studies (Tokşen, 2007; Er&Şevki, 2015; Özak et al., 2016; Yavuzcan Yıldız & Korkmaz, 2021), however, this parasite has not been studied as a welfare biomarker till now. Yet, the easy visibility of Lernantropus to the naked eye may provide the basis for its potential use as a biomarker for European sea bass.

The development and implementation of non-invasive fish welfare biomarkers are essential for ensuring the ethical treatment of fish, promoting sustainable practices in the aquaculture industry, and enhancing our understanding of fish welfare in various settings. Despite the extensive attempts made towards the authentication of welfare indicators that are specific to each species, the identification of suitable combinations of these measures still proves to be limited for the majority of farmed species as stated by Magalhães et al. (2020).

In this study, the aim was to validate the potential use of visible gill parasites (*L. kroyeri*) as the non-invasive biomarker of fish welfare by correlating them with key stress parameters such as hematocrit, blood glucose, and lactate.

MATERIAL AND METHODS

The fish examination was done immediately after harvesting on a commercial farm in the Aegean Sea. Fish samples ($N = 37$) were randomly selected among the harvested batch from one cage in the autumn. The weight of European sea bass (*D.labrax*) was around 350 g. During the harvesting period, the water temperature was measured as 23 °C and the dissolved oxygen level as 6.5 mg/L.

Blood samples were collected promptly upon the taking of the fish out. Prior to blood sampling, the fish underwent a brief anesthetic procedure involving a clove oil solution (5 mg/L) for a duration of 5 minutes. Heparinized syringes were utilized to collect blood via the cardiac puncture. Hematocrit measurements were conducted immediately following blood sampling. Plasma was obtained by centrifugation of heparinized blood at 3000*g* for 10 min and pooled where necessary. The plasma was then preserved at a temperature of -18°C until further analysis. Commercial kits (Cayman Chemicals, USA) and a Shimadzu UV-1210V spectrophotometer were used to measure plasma glucose and lactate, in accordance with the manufacturer›s instructions.

The copepod parasite *L. kroyeri* was carefully plucked from the gills by using forceps in the newly harvested fish (Figure 1). Parasites on both sides of the gills were counted on the gills with the naked eye (Figure 2). The sex of the parasite was not considered.

Statistical analyses: The variation in stress parameters was examined using an ANOVA test based on parasite counts. Regression analyses were conducted to test whether there is a significant relationship between parasite counts on the gills and secondary stress parameters (hematocrit, plasma glucose and lactate).

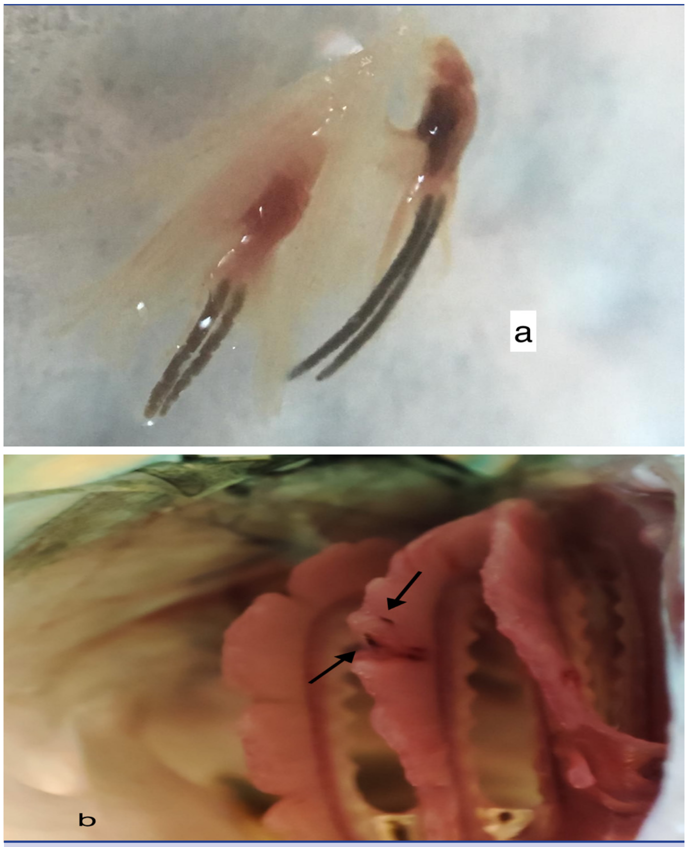

Figure 1. a) Copepod parasite, *Lernantropus kroyeri* b) *L. kroyeri* on the gills of European sea bass.

RESULTS AND DISCUSSION

In this study, the validation of visible ectoparasites (crustacean parasite, *L. kroyeri*) on the fish gills as a non-invasive fish welfare biomarker has been evaluated. The welfare status of the fish was assessed using the stress indicators as the concept of stress is closely connected to that of fish well-being (Galhardo & Oliveira, 2009). When an organism's coping mechanisms fail to adapt to its environment adequately, it leads to poor welfare (Broom, 2008). Stress can negatively impact fish health, growth, reproduction, and overall well-being of fish.

The prevalence of *L. kroyeri* on sea bass gills was 78.37%. Stress indicators (hematocrit, plasma glucose, and lactate) showed significant differences by the parasite *L. kroyeri* number in the gills of the fish ($p < 0.05$). The variation in the stress indicators was parasite number-dependent (Table 1).

Wells & Pankhurst (1999) noted that the persistence of altered states of glucose and lactate as well as hematological changes following stress are clearly-defined indicators of the stress response although the variations in these indicators may be considered high to observe chronic stress (Magalhães et al., 2020). The increased hematocrit is an indication of the increase in the oxygen requirement of the fish body. Under stressful circumstances, there exists a possibility for hematocrit values to exhibit an elevation, which is a compensatory mechanism aimed at augmenting the supply of oxygen to the organs as a response to the escalated metabolic demand (Fazio et al., 2015). Increased hematocrit levels have been reported for the hypoxic conditions in sea bream (Bermejo-Nogales et al 2014). Thus, here, the higher levels of hematocrit in sea bass with higher numbers of *L. kroyeri* can be explained by the disrupted oxygen balance in the gills. The increase in glucose and lactic acid levels in fish during stress reflects their physiological response to challenging conditions (Levy de Carvalho Gomes, 2007). These metabolic changes help the fish adapt to the stressor, providing the necessary energy for survival, but prolonged or severe stress can have negative effects on fish health and welfare (Wells & Pankhurst, 1999). Blood glucose serves as the primary energy fuel in stressful conditions (Wendelaar-Bonga, 1997) and blood lactate is produced as a metabolic byproduct during certain physiological conditions, including stress (Schreck & Tort, 2016). However, high blood lactate can limit oxygen transport to the tissues in fish (Olsen et al., 1992). Plasma glucose and lactate increased by the parasite numbers on the gills in a manner that is parasite number-dependent in this study. Here, the increase in parasite numbers being associated with an increase in hematocrit, plasma glucose, and lactate levels is an important finding of this study.

Table 1. The changes of stress indicators in sea bass by the *L. kroyeri* intensity.

***L. kroyeri* intensity in the gills**	**Frequency of parasitized fish (N_{total}=37)**	**Hematocrit (%)**	**Plasma glucose (mg/dL)**	**Plasma lactate (mg/dL)**
0 (uninfested fish)	8	30.62*	67*	10.25*
1-3	9	33	83.88	15.33
4-10	11	35	91.72	17.18
>11	9	36.11	129.11	17.44

*refers the difference for the values in the column

The regression analysis resulted in a significant overall effect of the independent variables (parasite intensity on the gills) on the dependent variables; stress indicators including hematocrit, plasma glucose, and plasma lactate (Table 2).

The regression results for hematocrit provides a moderate-to-good explanation of the hematocrit variability (R^2=0.56 and F=44.75), however, the individual regression coefficient is statistically significant ($p<0.01$), suggesting that parasite number has some predictive power for hematocrit values of sea bass (Figure 2).

For plasma glucose, the regression model appeared to be highly significant (R^2=0.85 and F=200.45), indicating that the ectoparasite numbers have a substantial impact on the plasma glucose of sea bass (Figure 3).

The regression model was statistically significant (F = 36.41) and explains a moderate amount of the variability in the plasma lactate (R^2=0.50), indicating that the model has identified significance between parasite intensity and plasma lactate (Figure 4).

Regarding the potential use of visible ectoparasite presence as a non-invasive biomarker, regression analyses have shown a strong relationship between changes in stress indicators and parasite numbers in the present study. The regression results provide strong evidence that the parasite numbers on the gills have a significant impact on the secondary stress parameters of hematocrit, plasma glucose, and plasma lactate. Based on these regression results, the presence and intensity of parasites can be utilized as a non-invasive biomarker for fish welfare assessment. Furthermore, to strengthen the argument for the usability of parasites as biomarkers, it should be emphasized that parasites serve as a biomarker indicating both fish welfare and environmental quality in aquaculture. The rise in parasite numbers in fish can serve as a warning sign of an unsuitable environment. Thus, a comprehensive approach has been implemented for addressing sea lice in salmon farming in Norway. Sea lice are regarded as indicators of regional environmental sustainability, and certain limitations on biomass production are imposed based on the recorded parasite levels on farms within a specific area. Farms that consistently maintain low parasite abundance on fish are permitted to produce larger biomasses (Bui et al., 2019). It is also essential to highlight their consistent presence and relevance in different ecological contexts. Copepod parasites such as *L. kroyeri* and their copepodites are available throughout the year, resulting in constant infection pressure in the sea cages (Yavuzcan Yildiz & Korkmaz 2021). The crustacean parasite's continuous presence provides suitability for using these parasites as non-invasive biomarkers.

Table 2. The key parametric in regression analysis for stress indicators.

Dependent variable*	R-squared	*F* value	*P* value
Hematocrit	0.56	44.75	<0.01
Plasma glucose	0.85	200.45	<0.01
Plasma lactate	0.50	36.41	<0.01

* The independent variable was the parasite number on the gills

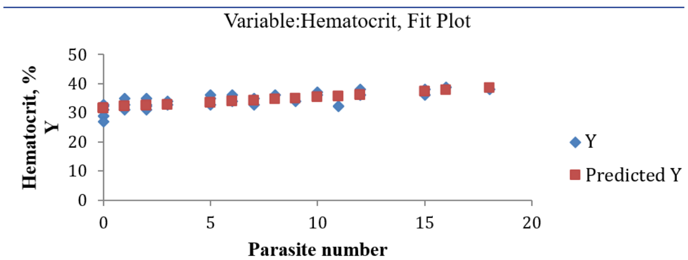

Figure 2. Hematocrit.

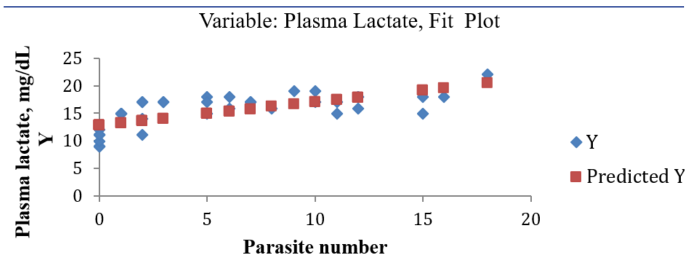

Figure 4. Plasma lactate.

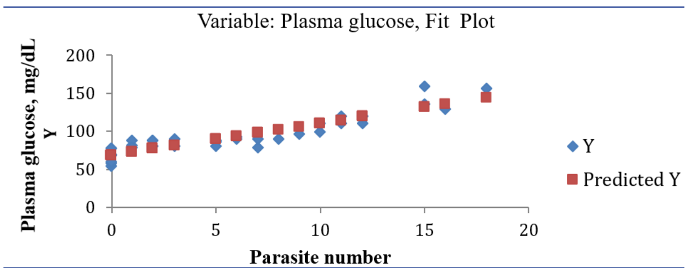

Figure 3. Plasma glucose.

CONCLUSION

While varying between moderate and strong, a statistically significant relationship exists between the numbers of copepod parasites (*L. kroyeri*) on the gills and secondary stress indicators (hematocrit, blood glucose, and lactate) in sea-caged European sea bass. In the regression analysis, R-squared values (approximately 50%) for the hematocrit and plasma lactate indicate that the independent variables in the model explain half of the variance in the dependent variable, necessitating cautious interpretation. An R-squared value of 0.85 is relatively high and suggests a strong positive linear relationship between the plasma glucose and the parasite number on the gills in the regression. Nevertheless, with R-squared values of each biomarker consistently above 50%, it is considered that the results of this study can support the utilization of parasite count as a non-invasive biomarker for fish welfare assessment. Therefore, the visible ectoparasite presence on the surface of the fish and their intensity can be suggested as a non-invasive biomarker for assessing fish welfare in aquaculture and an effective tool in explaining the welfare status of the fish. The validation process typically included defining requirements, establishing testing protocols, conducting tests, analyzing results, and documenting the findings. The goal was to provide confidence that the presence of visible parasites is fit to welfare assessment as a non-invasive biomarker, however, both validation and verification would be essential steps in the integration of visible parasites on fish gills in operational welfare indicators as the non-invasive biomarker.

It's worth noting that the development and validation of non-invasive biomarkers for fish welfare are ongoing research areas. Exploring new techniques and biomarkers to improve the assessment of fish welfare while minimizing the impact on the fish is significant for sustainable aquaculture.

Conflict of interest: The author declares no conflict of interest.

Ethics committee approval: Ethical approval for this study was obtained from ANKARA UNIVERSITY (2018-16-101). The present study followed international, national, and/or institutional guidelines for humane animal treatment.

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