UNIVERSITY OF WARMIA AND MAZURY IN OLSZTYN

Polish Journal of Natural Sciences

37

(2/2022)

PUBLISHER UWM
OLSZTYN 2022

The Polish Journal of Natural Sciences is indexed and abstracted
in Biological Abstracts and Biosis Previews

The print edition is the primary version of the Journal

The Journal is also available in electronic form on the websites
http://www.uwm.edu.pl/polish-journal/ (home page)
https://czasopisma.uwm.edu.pl/index.php/pjns/about (electronic platform; submissions)

PL ISSN 1643-9953

Publisher UWM Olsztyn
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Edition 75 copies; publisher's sheets 9.85; number of printed sheets 8.0
Print Zakład Poligraficzny UWM w Olsztynie
order number 631

TABLE OF CONTENTS

Animal Breeding and Production

Biology and Forestry

Fisheries

Food Sciences

POLISH JOURNAL OF NATURAL SCIENCES
Abbrev.: Pol. J. Natur. Sc., Vol 37(2): 153–165, Y. 2022

DOI: 10.31648/pjns.7795

THE APPLICABILITY OF INFRARED THERMOGRAPHY IN DEER FARMING

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Key words: thermogram, deer farming, fallow deer, veterinary diagnostics, animal welfare.

Abstract

The popularity of deer farming has increased in recent years. However, research into dedicated methods for diagnosing deer in view of their specific behavior, including low levels of domestication and high susceptibility to stress, is still scant. Infrared thermography could be a useful tool for assessing the behavior of farmed animals, including deer. This non-invasive diagnostic method has numerous applications, and it could facilitate farming operations without compromising the animals' welfare. Therefore, the aim of this study was to assess the applicability of infrared thermography in deer farming and to identify breeding practices where thermal imaging can be effectively applied.

Introduction

Infrared thermography is a non-invasive diagnostic method that relies on electromagnetic radiation sensors. Thermographic cameras register and process infrared emissions into graphic images presenting surface temperature distribution (SPEAKMAN and WARD 1998). Infrared radiation can be used for remote sensing of animals (IJICHI et al. 2019, SALLES et al. 2017, MCCAFFERTY 2007). This method can be a valuable tool for examining livestock and wild animals without premedication (POTRAPELUK et al. 2021). However, the applicability of infrared thermography for diagnosing farmed deer species such as red deer (*Cervus elaphus*), sika deer (*Cervus nippon*) or fallow deer (*Dama dama*) has not been thoroughly investigated to date. The growing popularity of deer farming poses new challenges for scientists who have to modify the existing research methods and expand the knowledge about the physiology and behavior of the farmed species.

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Deer are not domesticated animals, which is why thermal imaging cameras can significantly facilitate daily farming operations as well as specialist diagnostics.

This article reviews the literature on thermographic measurements in various species of farm and wild-living animals with the aim of formulating methodological assumptions for the use of thermal imaging as a diagnostic tool in deer farms.

Observation and localization of deer in grazing paddocks

Animal welfare should be the most important consideration in every farm. In deer farms, the layout of all farm facilities, including paddocks, should be adapted to the animals' needs. Grazing paddocks should feature clusters of trees and shrubs where deer can rest and hide (JANISZEWSKI et al. 2016). However, such visual obstacles complicate animal observations.

OISHI et al. (2018) developed a method for monitoring the presence and movement of wild animals based on thermograms. The proposed method was tested in a relatively large area. Thermal images revealed the presence of 24 objects that had not been detected during a local survey. The study demonstrated that infrared cameras can be helpful in localizing individual animals as well as entire groups. However, the cited authors concluded that further research was needed to define the optimal conditions for thermographic observations.

Similar conclusions were formulated by MATSUURA et al. (2017) who relied on infrared thermography to identify wild sika deer camouflaged by vegetation. Measurements were conducted early in the morning. Differences were noted in the number of animals that were identified with and without an infrared camera. The study demonstrated that thermal imaging considerably improves and facilitates the search for individual animals and their groups without causing disturbance. These conclusions were validated by MCCAFERTY (2007) who analyzed the usefulness of infrared thermography in a study of livestock and wild-living animals. According to the cited author, thermography is a non-invasive diagnostic tool with a growing range of applications. Measurements made at a distance of less than 1 m supported examinations of specific sites of heat loss, whereas images captured over distances greater than 1000 m enabled the researcher to count large mammals. However, thermography has certain limitations in open areas where surface temperatures are influenced by solar radiation, humidity and evaporative cooling. To minimize these risks, measurements should be conducted at night or on cloudy days with low insolation.

FAYE et al. (2016) provided interesting insights about thermographic measurements in deer farms. They observed that distance to the measured object affects the reliability of thermographic images. The cited authors formulated general guidelines for ecological studies to minimize inaccuracies resulting from distance to the examined surface.

Thermal imaging can be used to monitor animal movement patterns as well as hiding behavior which is particularly common in young animals in the first weeks of their life (JANISZEWSKI et al. 2016). These behaviors can lead to injury or even death in wild-living animals. CUKOR et al. (2019) analyzed the effectiveness of thermography in reducing the mortality of roe deer fawns. Hiding deer were identified with an infrared camera carried by an unmanned aerial vehicle (UAV). Fawns were detected with 100% accuracy. The authors noted that the reliability of thermal imaging can be affected by the height of flight (the optimal height was estimated at 40 m), land form and time of day.

Unmanned aerial vehicles carrying thermographic cameras can be a useful tool for monitoring the behavior of farmed deer, but drones move rapidly and emit noise that could generate additional stress for the animals. It should also be noted that fallow deer fawns tend to hide behind nettles and small shrubs (JANISZEWSKI et al. 2016) that grow in clusters to a height of up to 1.5 m. However, the study by CUKOR et al. (2019) was conducted in alfalfa fields; therefore, the proposed method where thermal imaging cameras were carried by UAVs flying at a height of 40 m should be modified to account for local conditions.

Body temperature analysis

Thermographic methods can be used to examine physiological parameters in animals (KASTBERGER and STACHL, 2003, STEWART et al. 2005, BARROS et al. 2016). Accurate measurements of body temperature are very difficult to perform in farmed deer. The examined animal has to be immobilized in a crush, but temperature can be measured only in upper parts of the body. Immobilization also generates considerable stress which can influence the results.

OKADA et al. (2013) analyzed factors that affect the accuracy of body temperature measurements in cattle, including hair, body surface temperature, surface temperature of the eyeball, rectum temperature and ambient temperature. The surface temperature of the flank was also measured at different time points after eating. The authors determined the optimal conditions for each measurement. They concluded that the dis-

tance between the thermographic camera and the object should be fixed, and the camera should be positioned at a 45° angle relative to the monitored object. Measurements should not be conducted in extreme cold or heat, direct sunshine, high humidity or wind. Thermographic images should be acquired under identical conditions, and hairless body parts can be used if this is not achievable. Factors that affect temperature measurements in animals were also investigated by Roy et al. (2020). They concluded that infrared thermography provides more reliable estimates of surface temperature when performed in a controlled environment. According to McCafferty (2007), variations in infrared emissivity, the animals' physiological responses and diseases can induce changes in temperature, and these potential sources of error should be taken into account. Despite these limitations, thermographic imaging supports non-invasive temperature measurements in farm and wild-living animals.

The cited studies indicate that infrared imaging can be applied in deer farms, but external factors such as ambient temperature and insolation, have to be considered when interpreting the results.

Salles et al. (2017) demonstrated that infrared thermography supports the identification of differences in body temperature resulting from varied proportions of fiber in ruminant diets. However, the observed differences were small, and further research is needed to establish the relationship between changes in body temperature and fermentation dynamics in ruminants. In deer, such a novel approach would require methodological modifications as well as alternative research studies to validate the results.

Deak at al. (2019) relied on digital infrared thermography to evaluate the impact of season and pregnancy stage on the temperature of different body parts in dairy cows. Considerable differences in temperature were noted between seasons and the stages of pregnancy. A significant correlation was found between pregnancy stage and the temperature of external reproductive organs, rectum, muzzle, eyeball and flank. These results contribute important information for deer farming, but seasonal changes in coat insulation should be taken into consideration in these animals.

Reproduction

The physiological processes associated with reproduction are difficult to examine and monitor in deer because these animals are not domesticated and are reared in extensive paddocks.

DHANASEKARAN et al. (2017) examined the applicability of thermal imaging for analyzing male fertility, diagnosing pregnancy and estrus, localizing animals, and evaluating the impact of environmental factors on the behavior of various farm animals. Reproductive problems, including infertility and stillbirth, are also encountered in deer farms. According to the cited authors, these risks can be minimized through thermographic evaluations. The impact of environmental conditions on the surface temperature of the examined objects was regarded as the main limitation of thermal imaging.

Similar conclusions were drawn by CILULKO et al. (2018) in a study investigating the applicability of thermography for estrus detection in farmed fallow deer (*Dama dama*). An increase in temperature was noted in the area of reproductive organs. The main limitations of the study were the distance from the measured object and the examined site. These findings indicate that thermal imaging can be applied in deer farms as well as in zoos where animals are semi-domesticated. OKADA et al. (2013), MCCAFERTY (2007) and DHANASEKARAN et al. (2017) emphasized that to maximize the reliability of the results, thermographic measurements should be conducted in indoor premises with constant temperature, humidity and lighting. According to CILULKO et al. (2018), estrus can be most reliably detected when thermographic measurements are conducted at a distance of 1 m, which implies that the researcher has to directly approach the animal. Therefore, the applicability of thermal imaging is reduced in wild and undomesticated animals.

The advantages and limitations of thermal imaging in animal breeding and reproduction were also discussed by DOMINO et al. (2022), DEAK et al. (2019), GEORGE and CHACUR (2017), DHANASEKARAN et al. (2017), SILVA et al. (2018) and RUEDIGER et al. (2016). Domino et al. (2022) relied on infrared thermography to detect estrus in horses. Heat emissions from body surfaces increased in successive stages of pregnancy due to an increase in blood flow and metabolic activity in uterine and fetal tissues. The resulting changes in temperature were captured in thermographic images. According to the authors, the developed digital image processing method supports early pregnancy detection in mares. In theory, the method described by DOMINO et al. (2022) could be used in deer farms, but the proposed approach would have to be modified to account for the specific characteristics of deer.

CILULKO et al. (2018) relied on thermal imaging to monitor the progression of pregnancy in farmed deer. They acquired thermograms of the lower abdomen and the rump (control area) in pregnant fallow deer does. The examined areas differed in temperature, and the greatest differences

were noted in the third trimester which is characterized by rapid fetal development. Thermal imaging facilitates pregnancy monitoring in fallow deer, but this method requires some degree of animal domestication. It should also be noted that fallow deer usually come into estrus in October, and offspring are born in June and July. Therefore, changes in coat insulation should be taken into account when monitoring the progression of pregnancy in this species. The winter coat is an effective insulator, which is why the temperature measured in the control area (rump) can differ from the actual skin temperature. Animal adaptations to environmental conditions can thus lead to differences in body temperature and measurement errors.

In deer farms, fawns and calves should be monitored in the early postpartum period. Such observations are conducted not only to assess the health and wellbeing of offspring, but also to localize hiding fawns and calves. CILULKO et al. (2018) demonstrated that thermal imaging is highly useful for monitoring young animals, but the results can be burdened with error. Thermograms are most reliable when measurements are conducted at a distance of up to 20 m to ensure that the heat emitted by the animal is clearly visualized. In deer farms, infrared measurements are performed outdoors; therefore, the influence of the time of day, insolation and ambient temperature should be taken into account.

Health assessments

According to WELLIGTON et al. (2019), infrared thermography is similar to a body scanner because it can be applied to visualize variations in body temperature and metabolic changes associated with the onset of inflammatory processes. Thermal imaging has been applied to assess the health of sheep (D'ALTERIO et al. 2011, SUTHERLAND et al. 2020, GELASAKIS et al. 2021), pigs (GRACIANO et al. 2014), cows (SCHAEFER et al. 2012), horses (KIM and CHO 2021a) and calves (LOWE et al. 2020).

Godyń (2013) demonstrated that thermography can be a useful tool for diagnosing limb health in livestock. The observed temperature anomalies were indictive of pathological changes. According to the cited author, the presented approach supports early detection of foot and limb disorders in animals. This diagnostic method could be also applied in deer farms, but the animals must be sufficiently tamed for the researcher to perform the measurements at close range.

SAMARA et al. (2014) investigated the applicability of infrared thermography for early detection of subclinical mastitis in dairy camels. They

concluded that this method is highly useful for distinguishing subclinical mastitic udders in lactating camels, which is crucial for early detection and effective treatment of the disease. Similar observations were made by RACEWICZ et al. (2018) who analyzed mastitis in dairy cows. However, the results of the cited studies are unlikely to have practical significance for deer farms due to udder location in hinds and does. In deer, the udder is localized in the region of the lower abdomen and reproductive organs; therefore, mammary gland temperature cannot be measured without direct human intervention, which is a source of considerable stress for the animal.

SCHAEFER et al. (2012) relied on an automated and non-invasive infrared thermography system to detect early stages of bovine respiratory disease in cattle. The system was installed around a water station that was accessible to all animals. Body temperature and drinking frequency were measured automatically without the need for direct animal handling. The study revealed that data collected non-invasively by the designed thermographic system were more reliable than the results of measurements that were performed on immobilized animals and caused stress. The cited authors also found that thermographic measurements of eye temperature facilitated diagnosis of respiratory disorders in calves.

Similar studies have not been conducted in deer farms which consist of outdoor paddocks. If a thermal imaging system were to be installed around water stations, the results could be influenced by environmental conditions. However, in the light of Polish regulations (Regulation of the Minister… Journal of Laws, 2004. No. 215, item 2188), red deer and fallow deer can be farmed indoors; therefore, health assessments based on thermographic measurements could be performed in a controlled environment.

DUNBAR et al. (2009) relied on infrared thermography as a rapid and non-invasive tool for assessing the health of deer. The study was conducted on mule deer (*Odocoileus hemionus*) experimentally infected with foot and mouth disease. Body temperature was significantly elevated in infected animals. The described method can be applied in the farm setting to identify early symptoms of infection and minimize production losses.

Antler development

Antler trimming is performed routinely in deer farms. Antlers are cut when male deer rub the velvet off the newly acquired antler growth and when antlers are completely ossified. The treatment must be appropriately timed to prevent pain. Trimming times are determined based on

observations of antler growth and development and testosterone levels in the blood (CHAPMAN and CHAPMAN 1975). In a study of red deer, BOWERS et al. (2010) observed an increase in velvet antler surface temperature measured with a thermographic camera. The rise in temperature was indicative of antler growth, which suggests that infrared thermography could be useful for monitoring this process.

POTRAPELUK et al. (2021) analyzed changes in velvet antler temperature and the timing of the velvet shedding period in fallow deer (*Dama dama*). The measurements were conducted at three points on the main beam. The stages and rate of antler growth were effectively captured by the obtained thermograms. This non-invasive method supported the determination of the velvet shedding period and the optimal antler cutting times. This method can be applied in deer farms and zoos to improve animal welfare and guarantee the safety of personnel during animal handling operations.

Behavior and stress

Deer are far less domesticated, more easily startled and more susceptible to stress that other farmed animals (JANISZEWSKI et al. 2016). For this reason, non-invasive thermal imaging methods could be particularly useful for monitoring deer behavior in farming practice (KASTBERGER and STACHL 2003).

Farmed deer have to be immobilized during routine operations such as antler trimming, administration of medications and microchipping. For this purpose, the animals have to be diverted from grazing paddocks to raceways and holding pens. This process involves considerably physical exertion and high levels of stress, which can compromise future carcass quality.

BARTOLOMÉ et al. (2021) conducted an interesting study into effort and muscle recovery in sport performance. During a performance test, the authors used a thermographic camera to measure eye temperature in sport horses. An analysis of the obtained thermograms revealed that the greater the increase in eye temperature, the higher the horse's position in the ranking. The cited authors concluded that thermographic measurements of eye temperature could be a useful method of assessing effort and recovery in animals. Similar observations were made by REDAELLI et al. (2019) who diagnosed physiological stress and documented the effects of endurance training in horses based on thermographic measurements as well as serum cortisol levels, blood counts and heart rate measurements.

The study demonstrated that thermography could be a useful non-invasive tool for evaluating physiological stress in animals.

These findings suggest that thermography can be applied as an additional method for assessing the health of deer, predicting weight gains and dressing percentage of primal cuts, evaluating the animals' susceptibility to stress and physical exertion during herding. However, thermographic methods have to be adapted to the tested species, and an adequate population sample has to be selected. According to REKANT et al. (2016), infrared thermography can replace other methods of disease detection in animals. It facilitates non-invasive health assessments, thus improving animal welfare and the farm's performance. Infrared thermography is a valuable diagnostic tool that continues to evolve and has a growing number of applications.

LOWE et al. (2020) observed that automated systems are increasingly used in the livestock industry. They designed an automated system for collecting thermal infrared images to monitor the health and welfare of calves. An infrared camera was integrated into an automated calf feeder. The authors developed an algorithm for automatically detecting and analyzing eye and cheek regions in thermographs. Data were collected automatically when the animals approached the feeder, with no human intervention. The authors concluded that thermography can be effectively used in cattle breeding, in particular for monitoring the health and welfare of calves.

In contrast, in the work of SUTHERLAND et al. (2020), infrared thermography was not superior to other diagnostic methods. The cited authors set out to determine whether eye temperature measured by infrared thermography is a reliable indicator of autonomic nervous system activity (ANS) in sheep. They concluded that heart rate variability was a more sensitive and effective method of measuring ANS activity than infrared thermography. The described approach would not be highly effective in deer farms due to low levels of domestication in red deer and fallow deer. In the described methodology, heart rate monitors were strapped around the thorax. When deer are immobilized in a crush, only selected parts of the upper body can be accessed; therefore, heart rate measurements cannot be conducted in the same manner. Prolonged immobilization would also increase stress levels and affect the reliability of the results.

As mentioned in the previous sections, thermograms are influenced by environmental conditions. IJICHI et al. (2019) investigated whether the position of an infrared camera impacts the recorded temperature and which camera positions are optimal for diagnosing stress responses in animals with anterolateral eyes. Thermographic images are commonly taken

at an angle of 90° relative to the eye. The authors suggested that a 90° angle relative to the sagittal plane was the optimal position for measuring eye temperature. The cited study offers a validated protocol for using infrared thermography to measure stress and welfare in animals with anterolateral eyes.

KIM and CHO (2021b) demonstrated that thermograms of the eye are useful indicators of welfare in horses. The authors attempted to determine the most appropriate areas for measuring eye temperature. The effects of camera calibration, distance and angle of measurement were taken into account. The authors concluded that further studies were needed to evaluate the applicability of eye temperature measurements for assessing pain and stress levels in horses.

Similar studies are planned in farmed deer to validate the usefulness of eye temperature measurements as reliable stress indicators. The developed measurement protocol could be applied to improve rearing standards and, above all, animal welfare in deer farms.

Conclusion

The presented literature review indicates that infrared thermography has numerous applications for analyzing physiological and behavioral processes in animals. Non-invasive diagnostic methods are increasingly popular in animal rearing. Deer differ from other farmed animals in terms of behavior and level of domestication, and these factors should be taken into consideration when developing remote diagnostic protocols for deer. In deer farms, infrared thermography should be applied mainly to assess animal welfare, including stress responses to routine husbandry practices.

Translated by ALEKSANDRA POPRAWSKA

Accepted for print 26.07.2022

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POLISH JOURNAL OF NATURAL SCIENCES
Abbrev.: Pol. J. Natur. Sc., Vol 37(2): 167–176, Y. 2022

DOI: 10.31648/pjns.7695

RESPONSE OF FARMED FALLOW DEER TO HUMAN PRESENCE

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Key words: stress, behaviour, *Dama dama*, deer farming.

Abstract

Direct behavioural responses to human presence are a very important consideration in red deer and fallow deer farming. Farm employees performing daily operations and visitors (agritourism) can trigger unexpected animal behaviours, elicit antagonistic responses or even contribute to accidents. Therefore, the aim of this study was to describe the behavioural responses of farm-raised fallow deer to the presence of strangers in the direct vicinity of the grazing paddock. Two independent behavioural tests were designed to analyse the responses of individual animals to a person or a group of people in the immediate vicinity. The results of a three-way analysis of variance revealed a significant main effect of the number of observers, which points to differences between groups, regardless of the animal's sex and observation date. Fallow deer were more agitated in the presence of individuals than groups of people. It is important for the maintenance of animal welfare, both during routine work on the farm and during the observation of their health.

Introduction

Deer farming differs from the production of other livestock species because red deer and fallow deer exhibit different behavioural responses and are more susceptible to stress (FLETCHER 2002, WILSON 2002). To reduce animal stress, deer farms should be well-designed and furnished, and fallow deer should be handled appropriately during routine farming operations in grazing paddocks and farm buildings (MATTIELLO 2009, JANISZEWSKI et al. 2008). These considerations play a particularly important role during routine farming operations and veterinary treatments,

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including feeding, handling, antler cutting (hard bone) and deworming (GREEK and STOWE 2000). For instance, a thorough knowledge of feeding behaviours and eating habits of different sex and age groups is needed to design effective feeding plans (MATTIELLO et al. 1997).

Research suggests that direct human presence induces negative physiological (stress) and behavioural (escape, anxiety) responses in animals (MACARTHUR et al. 1982, GEIST et al. 1985, RUSHEN et al. 1999). Animals subjected to chronic stress are more susceptible to disease (BULLOCK et al. 1993) because high corticosteroid levels decrease immunity (BROOM and JOHNSON 1993) and energy expenditure increases when animals are startled and disturbed (MACARTHUR et al. 1992). Stress can also compromise livestock performance and the quality of the end product (HEMSWORTH 2003), and it can lead to quality defects in venison which is characterised by high nutritional value. The presence of other livestock species in neighbouring enclosures can also undermine the welfare of farmed cervids (ABEYESINGHE and GODDARD 1998).

Human presence can be a source of stress, and it can lead to aggressive responses in farmed red and fallow deer (CARRAGHER et al. 1997). Farm employees, veterinarians, etc. performing daily operations and visitors (agritourism) can trigger unexpected animal behaviours, elicit antagonistic responses or even contribute to accidents. Therefore, the aim of this study was to describe the behavioural responses of farm-raised fallow deer to the presence of strangers in the direct vicinity of the paddock.

Material and Methods

The study involved 15 fallow deer (8 females and 7 males) aged about 13 months at the beginning of the experiment. The animals were separated from the herd and located in an experimental paddock. The animals were previously placed in a large paddock with other individuals of farmed herd, among others with their hinds. This ensured that in the group of animals subjected to experimental observations, there was no strong social structure, which could interfere with the results. The experimental paddock had an estimated area of 2.0 ha, and it was surrounded by a secure fence that prevented the animals from escaping.

Video footage recorded by 10 camera traps and a digital video camera (x 20 optical zoom) was analysed. Camera traps were distributed to cover the entire area of the experimental paddock. The camera operator remained hidden in an observation tower outside the experimental paddock. The location of the observation tower and entry and exit times were carefully planned to avoid any disruptions in the animals' behaviour.

The aim of behavioural tests was to determine the animals' responses to the presence of a stranger or a group of strangers (observers) talking in a "normal" voice outside the paddock in the immediate vicinity of the fence.

Each day, observations and video recording began at 5.30 p.m. and ended at 7.30 p.m. At around 6.00 p.m., when standard farming operations had been completed and the animals were calm, an observer/observers slowly approached the experimental enclosure, paused for approximately 30 seconds by the fence and looked at the animals. The behaviour of the observer/observers was identical each time. Fallow deer's responses were monitored from the moment the observer/observers approached the fence until they walked away and disappeared from sight.

Two independent behavioural tests were designed to analyse the responses of individual animals to a person or a group of people in the immediate vicinity of the paddock. The animals' responses were evaluated on an 8-point scale in each test, where 1 point denoted the most desirable behaviours, and 8 points denoted the least desirable behaviours in fallow deer farming (Table 1).

Table 1

Description of scores in behavioural tests

Score	Test 1 – one observer	Test 2 – group of observers
	description	description
1	the animal completely ignores the observer	the animal completely ignores the observers
2	the animal raises/turns its head, gazes at the approaching observer, but does not walk away to a different part of the paddock	the animal raises/turns its head when it hears the approaching observers, but does not walk away to a different part of the paddock
3	the animal raises/turns its head, gazes at the approaching observer and after a while, follows other retreating animals and walks away to a different part of the paddock	the animal raises/turns its head, gazes at the approaching observes and after a while, follows other retreating animals and walks away to a different part of the paddock
4	the animal raises/turns its head, gazes at the approaching observer and after a while, calmly walks away from the fence	the animal raises/turns its head, gazes at the approaching observers and after a while, calmly walks away from the fence
5	the animal gazes at the observer and runs away together with other animals when the observer approaches the fence	the animal gazes at the observers and runs away together with other animals when the observers approach the fence
6	the animal gazes at the observer and runs away when the observer approach-es the fence	the animal gazes at the observers and runs away when the observers approach the fence

cont. Table 1

7	the animal walks away from the fence when other animals spot the observer and walk/run away	the animal walks away from the fence when other animals spot the observers and run away
8	the animal runs away from the fence immediately after spotting the approaching observer	the animal runs away from the fence immediately after spotting or hearing the approaching observers

The experiment began on 2 July 2020 and ended on 7 September 2020. Fallow deer were monitored and recorded during the entire experimental period. Each behavioural test was conducted in ten replicates at weekly intervals. The tests were performed alternately at intervals of several days to rule out any interactions between the results. The dates on which each test was performed during the experiment are presented in Table 2.

Table 2

Dates on which behavioural tests were performed during the experiment (year 2020)

Observation	1	2	3	4	5	6	7	8	9	10
Test 1	6.07	13.07	20.07	27.07	3.08	10.08	17.08	24.08	31.08	7.09
Test 2	2.07	9.07	16.07	23.07	30.07	6.08	13.08	20.08	27.08	3.09

Because the study relied on observations only, without direct contact with the man was not needed the consent of the Local Ethical Committee.

The results were processed with the use of three-way mixed model analysis of variance with 2 x 2 x 10 factorial design. The results were regarded as statistically significant at $\alpha = 0.05$. Statistical analyses were performed in the IBM SPSS Statistics 26 package.

Results

The effects of observation date, number of observers and the animal's sex on fallow deer responses were determined by three-way mixed model ANOVA with 2 (between-subjects factor – observer: one person vs. a group of persons) x 2 (between-subjects factor – animal's sex: male vs. female) x 10 (within-subjects factor – observation date: 1 to 10) factorial design. The results of the analysis are presented in Table 3.

Table 3

The results of three-way mixed model ANOVA with 2 x 2 x 10 factorial design testing the effects of observation date, number of observers and the animals' sex on fallow deer responses

Item	MS	df	*F*	*P*	η^2
Observation	4.08	9.234	16.30	< 0.001	0.385
Number of observers	25.38	1.26	66.79	< 0.001	0.720
Animal's sex	0.03	1.26	0.08	0.776	0.003
Number of observers * animal's sex	0.22	1.26	0.58	0.452	0.022
Observation * number of observers	4.15	9.234	16.58	< 0.001	0.389
Observation * animal's sex	0.18	9.234	0.70	0.705	0.026
Observation * number of observers * animal's sex	0.18	9.234	0.70	0.707	0.026

The analysis revealed that the number of observers was a significant main effect, which points to differences between groups, regardless of the animal's sex and observation date. Fallow deer were more agitated in the presence of an individual observer (M = 5.84; SD = 1.59) than a group of observers (M = 5.25; SD = 2.17). This factor explained 72% of the variance of the dependent variable (η^2 = 0.72).

The effect of observation date was also statistically significant, which implies that stress levels (mean value of the test result) differed across observation dates regardless of the number of observers and the animal's sex. This difference explained 38.5% of the variance of the dependent variable (η^2 = 0.385). The Bonferroni pairwise comparison test revealed several dozen significant differences. These differences cannot be fully described due to space constraints, but they demonstrated growing levels of stress in the studied fallow deer. The animals were most apprehensive during the last three observations and during observations No. 5 and 6. The descriptive statistics for each observation are presented in Figure 1.

The interaction effect of the number of observers and observation date was also statistically significant, which facilitates an interpretation of the differences in stress levels. An analysis of simple effects demonstrated that stress levels remained unchanged in the presence of a group of observers (the results of the post-hoc test for all observations were not significant). In turn, differences were noted when a single observer approached the fence. Several dozen differences were also observed, and mean values were highest during the last three observations, followed by observations No. 5 and 6. The descriptive statistics for this interaction effect are presented in Figure 1.

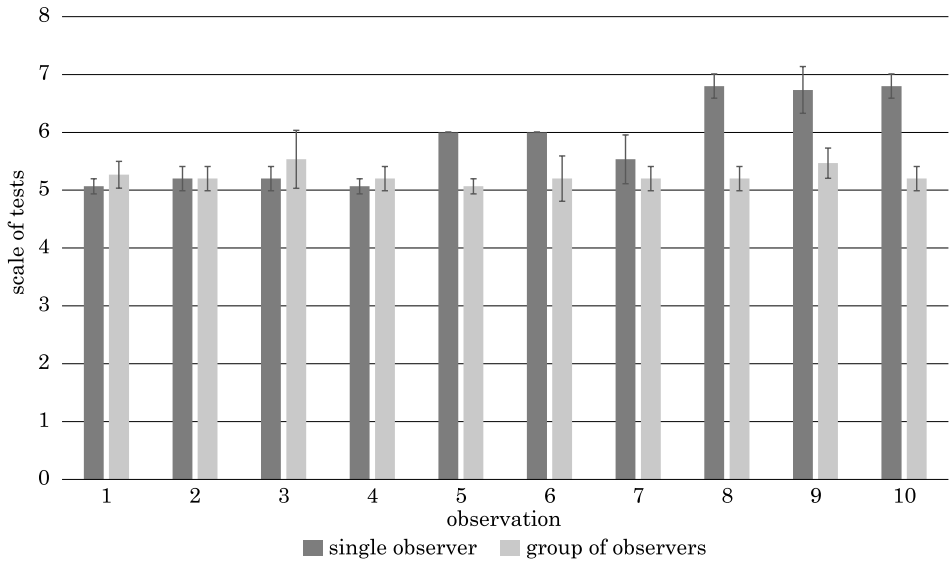

Fig. 1. Mean values and 95% confidence intervals for stress levels in fallow deer on each observation date, depending on the number of observers

As shown in Figure 1, when fallow deer were observed by a group of people, the mean value of the behavioural test ranged from 5 to 6 points during the entire experiment (10 weeks). When a single observer approached the fence, the mean value of the behavioural test exceeded 6 points on several occasions, and it was close to 7 points at the end of the experimental period. These scores imply that fallow deer fled from the fence when they spotted an approaching individual, but they calmly observed an approaching group of people and fled only when the observers reached the fence line. These responses were not affected by the animals' sex.

Discussion

Various definitions and methods have been proposed to measure temperament, including susceptibility to stress or anxiety, in livestock (BURROW 1997). However, most definitions rely on observations of animal responses to the presence of farm employees or strangers. Observations of livestock behaviour should be performed routinely in daily farming practice. Research studies have demonstrated that daily interactions between breeders and animals can compromise productivity and animal welfare (CARRAGHER and MATTHEWS 1996). Despite the fact that many interactions and handling operations occur routinely, some of them can be

a source of stress and anxiety, which was also indirectly confirmed by the results of the present study. Stress-inducing farming operations should be identified, and human-animal interactions that produce positive responses should also be analysed to minimise the negative consequences of handling operations that are necessary in livestock production (HEMSWORTH 2003). Behavioural observations also support the identification and assessment of pain in individual animals (STAFFORD and MELLOR 2002).

Red deer and fallow deer are characterised by low levels of domestication (FLETCHER 2020), which is why stressors and factors that elicit undesirable responses should be minimised in deer farms. Animal temperament and behaviour are influenced by both genetic and environmental factors (HEARNSHAW and MORRIS 1984), including breeding practices, handling experience and the behaviour of farm employees. The temperament of farmed deer and negative anthropogenic factors should be thoroughly analysed because fear, aggression and stress exert an adverse impact not only on meat quality, but also compromise the safety of humans and other farm animals (RUSHEN et al. 1999). SCHÜTZ et al. (2016) proposed a system for assessing the behaviour (temperament) of farm-raised red deer (hinds and their offspring) based on the following key traits: aggression, agitation in a pen or when confined in a crate, ease of handling, and exit speed from a crate. The results of the current study indicate that an animal's response to the presence of an individual or a group of people is also an important criterion in assessing the temperament of farmed red deer and fallow deer. The study demonstrated that fallow deer were more agitated in the presence of one person than a group of observers.

Animal responses to external factors, including stress factors, can be passed onto the offspring, which has significant implications for breeding practice, for example during the selection of breeding stock. In farmed deer, traits such as apprehension and anxiety can be passed from the mother to the offspring not only genetically, but also through imitation and imprinting (SCHÜTZ et al. 2016). Temperament is a trait with low heritability, but animal behaviours and responses to human presence should be taken into account in deer farming programs because calm deer are easier and safer to handle, and they grow more rapidly than hyperactive and agitated animals (WARD et al. 2019).

According to POLLARD (1983), animal welfare standards can be improved based on regular observations of the behaviour of farmed red deer. Changes in animal behaviour could be indicative of new stress factors or the elimination of the existing stressors. The cited author also suggested that individual responses to external factors associated with breeding and daily farming operations constitute valuable inputs for selective

breeding in deer farms. GRIGOR et al. (1998) examined the responses of one-year-old red deer hinds in five situations: transport, immobilization in a crush, human presence, visual isolation from the herd, and free escape (control group). Stress responses were assessed based on how fast the animals entered a narrow race in a handling pen. The highest stress levels were reported after 5 minutes of immobilization in a crush and after 5 minutes of transport. Hinds that were not subjected to other stressful procedures entered the race most quickly. However, the cited authors did not note the effect of employee behaviours or handling procedures (which should be standardised) on the responses of different animal groups.

Animal responses to stress factors can differ subject to individual experiences and perceptions of safety in the surrounding environment. The habituation of behavioural reactions occurs in response to frequent and predictable disruptions that are repeated, often localised and can be avoided (GEIST et al. 1985). According to BULLOCK et al. (1993), deer kept in parks can become accustomed to the presence of humans, which decreases the stress associated with human interactions. Behavioural responses to disturbances can also be analysed based on other factors that determine the animals' ability to escape from a potential threat. Stress responses can be reduced when animals have higher perceptions of safety, for example in large herds or in locations with an easy escape route (MACARTHUR et al. 1982). However, the "habituation" effect was not confirmed in the present study, where stress responses to the presence of individual observers were intensified in the second part of the experimental period (Fig. 1). It should be noted that the studied animals had had previous contact with farm employees, but routine farm operations differed from the observer's/observers' behaviour during the experiment.

MANTEUFFEL et al. (2009) hypothesised that the use of rewards in the process of training animals to perform simple tasks can improve animal welfare, decrease fear of humans and facilitate animal handling. Animals that learn to approach the trainer when given a visual or verbal cue can be rewarded with food. Individual animals can be regularly and frequently called during eating. Individual cows or groups of cows can be rewarded for coming to the milking unit in response to a particular command. Horses kept in boxes can be shown patterns on a screen before they are fed oats. According to the cited authors, animals that are trained to positively respond to specific situations will be less stressed by changes in their environment or handling operations. In the current study, the presence of an observer or a group of observers near the experimental paddock did not elicit a positive response.

It is difficult to explain why a single person approaching a fence caused a more stressful reaction to a fallow deer on a farm than a group of observers approaching. Perhaps the basis of this phenomenon should be the social structure of animals and permanent living in groups (CHAPMAN and CHAPMAN 1975). As noted in the Material and Methods chapter, young fallow deer over 13 months of age were involved in the behavioural experiment. Thus, they constantly lived in smaller or larger herds, and the presence of a single "external" individual was something new to them, and thus – stressful. However, this is only a hypothesis that should be subjected to further scientific analysis based on analogous studies of a larger number of farmer fallow deer herds.

HEMSWORTH (2003) argued that cognitive and behavioural training should be organised for animal breeders in the livestock industry. The ability to observe and interpret animal behaviours can be valuable not only in the process of recruiting farm employees, but also in discriminating between experienced and inexperienced workers that should be trained. According to the above author, extensive research is needed to identify the full range of interactions between farm employees that affect livestock.

In conclusions, the present study demonstrated that the presence of strangers in the vicinity of a grazing paddock is a stressful experience for farmed fallow deer. Regardless of sex, the animals were more inclined to run away from the fence when a single observer rather than a group of three observers approached the paddock, and this effect was intensified over time. Therefore, to minimise stress, the farm should have a layout that prevents strangers from approaching the animals. The response of farmed fallow deer to the close presence of individuals or groups of people can be also regarded as an important criterion in assessments of animal temperament and welfare in deer farms.

Translated by Aleksandra Poprawska

Accepted for print 8.06.2022

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POLISH JOURNAL OF NATURAL SCIENCES
Abbrev.: Pol. J. Natur. Sc., Vol 37(2): 177–186, Y. 2022

DOI: 10.31648/pjns.8110

EVALUATION OF BACTERIA BINDING POTENTIAL OF HAEMOLYMPH FROM TWO SPECIES OF GIANT AFRICAN LAND SNAILS (*ARCHACHATINA MARGINATA* AND *ACHATINA ACHATINA*)*

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Key words: giant African land snails, haemolymph, *Canavalia ensiformis,* lectin, bacteria binding.

Abstract

A study was conducted to evaluate the bacteria binding potential of haemolymph of two species of giant African land snails (*Archachatina marginata* and *Achatina achatina*) to four species of bacteria isolates (*Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa* and *Salmonella gallinarum*). Three liveweight groups (< 100 g, 101–150 g and > 150 g) were used for this study. Haemolymph aseptically collected from each liveweight group were incubated at 37ºC for one hour after which samples were examined under microscope for binding. Result obtained showed that binding pattern differ across the three liveweight groups with highest bacteria binding recorded at > 150 g. It can be concluded that lectin-like substance which agglutinate bacteria are present in the haemolymph of both species and that *Archachatina marginata* showed better binding potential considering livewieght group > 150 g. It can be concluded that substance (s) which agglutinate bacteria are present in the haemolymph of both specie.

Introduction

Giant African land snails are known to be found in the forest and moist area especially in plantations like cocoa, cola and plantain where they could obtain shade and moisture. Molluscs generally possess a natural

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* This research was supported financially by Innovators of Tomorrow STEP-B Award of the World Bank Project in Nigeria (HME/STEP-B/IOT/33/Vol 1/24) 2010.

immunity and anatomical structure that protect their soft tissue, body fluid losses and infections of pathogenic microorganisms and parasites (GLINSKI and JAROSZ 1997). As the snails go about their normal activity in their environment, there are tendencies of been constantly challenged by different types of microbial population which may hamper their normal body mechanism if not checked. The big question is 'how do they survive'? It means this animal must have a mechanism which enables them to get rid or control the invaders. DWEK et. al. (2001) revealed that this animal has agglutinin (Lectin) which has binding ability to sugar (oligosaccharides) and as such has been used as prognostic indicator for some cancers. ABIONA et. al. (2009) in his preliminary study identified the presence of agglutinins in the haemolymph of Giant African Land snails (*A. marginata* and *A. achatina*). Haemagglutination potential in both species to four different erythrocyte sources was also carried out to further evaluate their binding abilities (ABIONA et. al. 2014).

The haemocyte content of the haemolymph of invertebrates have been reported to play key roles in both cellular and humoral immune reactions by phagocytosis or delivering immune factors such as lectin and anti-microbial peptides (KANG et. al. 2006) It was further verified that bacterial infection causes changes in components such as lectins, anti-bacterial peptides and lysosomal enzymes of plasma or haemolymph in molluscs (KANG et. al. 2006). However, these component found in haemolymph have been reported to have therapeutic potentials (YANG et. al. 2008). The ability to produce copious amount of these component will determine to larger extent the survivability of this animal in their environment. The two most common species found in Western part of Nigeria are *Archachatina marginata* and *Achatina achatina.* However, their survivability differs especially to different environmental challenges. Other factor such as temperature may also be implicated for this reason but ability to constantly clear opportunistic infection in their system will make them survive better. CHRISTOPER (1980) reported that bacterial (*Pseudomonas aeruginosa*) which can induce elevated immunity in the foreign species of snail (Helix) is agglutinated by snail plasma. In this respect the animal will survive from enormous population of such bacterial. It therefore becomes important to evaluate the binding potential of the two common giant African land snails (*Archachatina marginata* and *Achatina achatina*) to four bacterial species to confirm presence of agglutinins and the quantity produced by white blood cells analoque known as hemocytes and other cellular components of the immune system in controlling the internal environment of the animal. This evaluation will further validate the presence of therapeutic agent in this animal and will also justify the usage of this animal for medical pur-

pose. Prompt recommendation could also be made to guide farmers who want to go into production with the sole aim of harvesting haemolymph/plasma for medicinal purpose on which species that will give the maximum yield at a particular stage of their growth.

Materials and Methods

Sample collection

About 5 ml of sterile snail haemolyph was aseptically collected from apex of three liveweight groups of giant African land snails (*Archachatina marginata* and *Achatina achatina*) after washing with distilled water and cleaning with methanol into a sterile universal bottle. Also, different clinical isolates of pure overnight broth suspension of *Eschericia coli*, *Salmonella gallinarum*, *Pseudomonas aeruginosa* and *Staphylococus aureus* were obtained from different veterinary diseased condition.

Qualitative bacteria agglutination

One milliliter (1 ml) each of overnight broth suspension of *E. coli*, *S. gallinarum*, *P. aeruginosa* and *S. aureus* (10^6 cfu/ml) was added to 1 ml of haemolymph collected aseptically from apex of three liveweight groups of giant African land snails (*A. marginata* and *A. achatina*) after washing with distilled water and cleaning with methanol. All metallic instruments used were gently heated and cooled before and after collection. Both haemolymph and bacteria samples were thoroughly mixed and incubated at 37°C for one hour in LTE incubator (Model UM040, LTE Scientific Ltd, UK). After incubation, the samples were microscopically examined for bound organisms and scored. This was then centrifuged to obtain the supernatant which was microscopically examined for unbound organisms using Centurion Scientific bucket centrifuge (Model M19, UK). The degree of aggregation/agglutination was estimated visually as follows:

- 4+: 80–100% of cells clumped (under a microscope);
- 3+: 60–70% of cells clumped (under a microscope);
- 2+: 30–40% of cells clumped (under a microscope);
- 1+: 10–20% of cells clumped (under a microscope).

Examination of bound and unbound organisms was carried out with three replicates of each of the sample used before arriving at the mean values used for qualitative agglutination count

Quantitative bacteria agglutination study

Supernatants obtained were serially diluted in phosphate buffered saline at ratio 1 : 10.1 ml of diluted sample at dilution of 10^6 bacteria was spread on Nutrient agar (Oxoid, UK) and incubated at 37°C overnight. Discrete colonies seen on the plates were counted. Total viable count of unbound bacteria cells in supernatants were calculated as described by MILES and MISTRAL (1938).

Total viable bacteria cell (unbound) in supernatant = $B \cdot 1 \cdot 1 \cdot 10^6$ cfu/ml.

Total count of bounded bacterial cells = 10^6 cfu/ml of broth – total viable count (unbound) in the supernatant.

Bacteria agglutination with lectin

Equal volume of 2 ml of 10^6cfu/ml bacteria isolates (*E. coli*, *S. aureus*, *Pseudomonas aeruginosa* and *S. gallinarum*) was added to 2 ml of various concentrations of standard lectin (*Canavalia ensiformis*) (1000 µg/ml, 800 µg/ml, 600 µg/ml and 400 µg/ml). They were thoroughly mixed, and incubated for 60 minutes at 37°C using LTE incubator (LTE Scientific UM040, UK). Each mixture was centrifuged at 3,500 rpm for 5 minutes using bucket centrifuge, and supernatant was aseptically removed. The supernatant was serially diluted and 1ml of $1/10^6$ dilution was spread on a well-dried Nutrient agar plate and allowed to set. The plates were incubated at 37°C for 18–24 hrs in a well aerated LTE incubator (LTE Scientific UM040, UK). Bacteria colony seen on the plates were counted using Biocote Colony Counter (Barloword Scientific, UK). The total viable count of each sample of the supernatant was calculated as follows:

Total viable count of unbound isolates obtained in the supernatant = $K \cdot 1 \cdot 10^6$.

Total viable count of the bound isolates = 10^6 cfu/ml – total viable count of unbound isolates obtained in the supernatant.

Results

Binding activity of haemolymph from three liveweight groups of two species of giant African land snails (*A. marginata* and *A. achatina*) is presented in Table 1. The large liveweight group (> 150 g) of *A. marginata* shown a very strong agglutination with *Staphylococcus aureus,* while *Escherichia coli* did not agglutinate with the haemolymph of medium liveweight group. Haemolymph from both species showed fair binding for *Pseudomonas aeruginosa* while *Salmonella gallinarum* was loosely agglutinated in both species. Table 2 shows binding activity of commercially prepared Lectin (Concanavalia A) with different clinical isolates. *Escherichia coli*, *Salmonella gallinarum*, *Staphylococcus aureus* had very strong binding with lectin at concentrations of 1000 µg/ml and 800 µg/ml before centrifugation (whole) while while *Staphylococcus aureus* and *Pseudomonas aeruginosa* showed loose binding at this concentration of 800 µg/ml in supernatant. The result of bacteria agglutination quantification is presented in Table 3. It was clear that at 400, 600, 800 and 1000 µg/ml, quantitative estimation of cell deposit after centrifugation increased with increase in lectin concentration, compared to cell number in the supernatant of the four bacteria isolates used except for *Escherichia coli* and *Pseudomonas aeruginosa* whose supernatant cell numbers were higher at 400 µg/ml (0.631 vs 0.369 cfu/ml and 0.51 vs 0.49 cfu/ml).

Table 1

Binding activity of haemolymph of three liveweight groups of giant African land snails (*Archachatina marginata* and *Achatina achatina*) to four species of bacteria

Specification	Snail species									
	marginata				PBS (-ve)	achatina				PBS (-ve)
	Bacteria isolates									
Liveweight group	ST	EC	PS	SM	–	ST	EC	PS	SM	–
Small	–	–	–	+	–	++	–	+	++	–
Medium	+	–	+	++	–	+	–	+	++	–
Large	+++	–	++	+	–	+	+	++	+	–

Key: ST – *Staphylococcus aureus;* EC – *Escherichia coli;* PS – *Pseudomonas aeruginosa;* SM – *Salmonella gallinarum;* PBS – *Phosphate buffer saline;* -ve – negative

+++*:* strong; ++: fair; +: loose

small: < 100 g; medium: 101–150 g; large: > 150 g

Table 2

Lectin (*Concanavalia A*) binding activity with different clinical isolates

Isolate	Whole				Supernatant				Lectin	+ haemolymph
									whole	supernatant
	lectin concentration [µg/ml]				lectin concentration [µg/ml]				[µg/ml]	
	1000	800	600	400	1000	800	600	400	800	800
ST	++++	++++	+++	+++	++	+	+	+	+++	++
EC	++++	+++	+++	++	+++	++	++	+	+++	++
SM	++++	+++	+++	++	++	++	+	+	+++	+
PS	+++	+++	++	++	+	+	+	+	+++	+

Key: ST – *Staphylococcus aureus*; EC – *Escherichia coli*; PS – *Pseudomonas aeruginosa*; SM – *Salmonella gallinarum*
++++: very strong; +++: strong; ++: fair; +: loose

Table 3

Lectin – bacteria agglutination quantification

Bacteria isolate	Lectin concentration [µg/ml]							
	400		600		800		1000	
	supernatant	deposit	supernatant	deposit	supernatant	deposit	supernatant	deposit
	($\cdot 10^6$ cfu/ml) (SEM)							
Staphylococcus aureus	0.412±0.001	0.588±0.001	0.233±0.0012	0.767±0.002	0.103±0.001	0.897±0.001	0.03±0.001	0.967±0.003
Eschericia coli	0.631±0.001	0.369±0.001	0.380±0.011	0.620±0.011	0.235±0.003	0.765±0.001	0.065±0.001	0.935±0.003
Pseudomonas aeruginosa	0.51±0.0115	0.49±0.0173	0.340±0.011	0.660±0.011	0.231±0.001	0.769±0.001	0.019±0.001	0.981±0.001
Salmonella gallinarum	0.48±0.0115	0.52±0.0115	0.280±0.023	0.720±0.006	0.106±0.001	0.894±0.001	0.035±0.003	0.965±0.001

Discussion

The agglutination potential of the agglutinins present in the haemolymph of different snail species (*Archachatina marginata* and *Achatina achatina)* against clinical bacteria isolates was demonstrated in this study. ABIONA et. al. (2014) had earlier reported the presence of agglutinins in these two snail species. For the small liveweight group (< 100 g), *Archachatina marginata* which showed no binding with *Staphylococcus aureus*, *Eschericia coli* and *Pseudomonas aeruginosa* may be as a result of lower proportion of lectins produced in this group which is in contrast with those

produced in *Achatina achatina* which agglutinated all the bacteria with the exception of *Eschericia coli*. The agglutination ability demonstrated by *Achatina achatina* of this group may be as a result of their level of sensitivity which may also be supported by their older age as compared to *Archachatina marginata* with lesser age due to their faster growth rate for this live weight group. It is quite understandable, the reason while age is playing prominent role in facilitating higher agglutination potential, since it takes times three of the age of *marginata* to obtain equal age with that of *Achatina*. Bindings/agglutination of bacteria by haemolymph which is said to contain lectin/lectin-like substance is an indication that certain sugar moieties present on the bacteria surface are the means of attachment for the lectin (KANG et. al. 2006) Also sugar types present on the surface at certain time also determines different lectin types which can bind them since lectins are sugar specific for their binding actions (PISTOLE 1981, MUNOZ-CREGO et. al. 1999). Consequently, the potential binding capacity of haemolymph from the three liveweight groups (small, medium and large) showed that pathogen surfaces bear a large number of oligoglucides that may be bound by specific lectins that can modulate host infection. According to MELHEM and VERDE (1984), adherence is said to be an important factor in bacteria colonization which is known to be supported by sugar carbohydrates of various forms. The medium liveweight group (101–150 g) in both species showed similar binding pattern. *Staphylococcus aureus* and *Pseudomonas aeruginosa* were loosely bound while *Salmonella* spp. was fairly bound. This may be an indication that lectin types which are specific for these bacteria types are present in both species at this liveweight. For *Escherichia coli* that were not bound by both species, it can be deduced that lectin or protein substance that can agglutinate the bacterium is not present in this group. For the large group (>150 g), *Archachatina marginata* haemolymph had a very strong binding with *Staphylococcus aureus* while loose binding was the case with *Achatina achatina*. This observation may be as a result of increased production of lectin specific type *for Staphylococcus aureus* or more protein substance which had the capacity to agglutinate this species of bacteria than *Achatina achatina*. *Pseudomonas aeruginosa* were fairly bound by both species. This is an indication that both species have the same binding ability for this bacterium. The same reason could be adduced for *Salmonella* spp. which was loosely bound by both *Archachatina marginata* and *Achatina achatina*. Loose binding demonstrated by the large group of *Achatina achatina* to *Eschericia coli* in this study is contrary to expectation, since *marginata* out-performed *Achatina* considering the four bacteria used in this study. This observation may be a pointer to the fact that few differences

occur in types of lectin produced. Several lectins have been shown to possess agglutination properties against bacteria strains (PISTOLE 1981, WATANABE et al. 2006, JIN et al. 2013, WANG et al. 2016, TEMPLIER et al. 2016, LV et al. 2016). However, where differences in sugars on the surface which act as attachment points for both micro-organism and lectin are noticed, binding will definitely be affected (SHERIFF 1994).

The results of lectin-binding pattern with the four species of bacteria at different concentrations showed that binding increase as the concentration increases for all the four bacteria strains. This observation is a further pointer to the fact that *Canavalia ensiformis* lectin (Con A) identified sugar moieties that it can bind on the surface of the bacteria. Con A is known to be mannose/glucose specific and as such aggregates a variety of gram-negative bacteria (LE MINOR 1973). Also, the sites of Con A binding are thought to be the exposed sugars of the bacteria lipopolysaccharide and the O-antigen factor 1 (LIENER 1976). So at higher lectin concentration more of lipopolysaccharide of the bacteria are exposed and occupied by available lectin and this explain the reason while binding increases with corresponding increase in lectin concentration. This observation is also in line with the report of MUNOZ-CREGO et. al. (1999) and SCHMID et. al. (2003) that pathogen surfaces bear a large number of oligoglucides that may be bound by specific lectins. The fact that Con A used in this study bind the four bacteria is a confirmation that this type of lectin is present in the haemolymph of giant African land snails (*Archachatina marginata* and *Achatina achatina*.

Considering the results of bacteria-agglutination quantification (Bacteria count), bacteria counted in coliform/ml were noted to increase as the concentration of the *Canavalia ensiformis* lectin increased (i.e 400, 600, 800 and 1000 µg/ml) across board for the four species of bacteria used. This progressive increase in binding was as a result of large amount of lectin available to occupy the binding sites available on the bacteria surfaces as the concentration increased. Also it could be inferred from this study that all the four bacteria have common sugars which are bound by this lectin type. According to AHMED (2005), *Canavalia ensiformis* lectin falls into D-mannose/D-glucose group of the plant lectin sources. This is a further confirmation that all the bacteria used have this sugar sequence on their surface which facilitated strong binding.

Acknowledgement

Authors thank members of staffs of Department of Veterinary Microbiology and Biotechnology Centre of Federal University of Agriculture, Abeokuta, Ogun State, Nigeria for their cooperation.

Accepted for print 5.02.2022

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POLISH JOURNAL OF NATURAL SCIENCES
Abbrev.: Pol. J. Natur. Sc., Vol 37(2): 187–204, Y. 2022

DOI: 10.31648/pjns.7538

CELLULASE PRODUCTION BY IMMOBILIZED CELLS OF *CANDIDA TROPICALIS* ISOLATED FROM GRASSHOPPER *ZONOCERUS VARIEGATUS* IN SAW DUST AND RICE HUSK MEDIUM

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Key words: immobilization, cellulase, *Zonocerus variegatus*, *C. tropicalis*.

Abstract

This study investigated the isolation of yeast from insect gut *Zonocerus variegatus*, screening and production of cellulase enzyme by immobilized yeast in saw dust and rice husk medium. Yeasts were isolated from the gut of grasshopper and were screened for cellulase production using Carboxyl Methyl Cellulose agar. Immobilization was carried out using *Irvingia gabonensis*. Effect of bead size, bead number, inoculum load and bead reusability were investigated. *Candida tropicalis* had the highest cellulase production, cellulase enzyme production was optimum at 72 h, 6 numbers of bead, 4 mm bead size, 6% gel concentration and 4% inoculum size. There was no obvious loss of activity with re-use of immobilized *Candida tropicalis*. This study shows that *C. tropicalis* isolated from *Zonocerus variegatus* can be immobilized on *I. gabonensis* and produce cellululase enzyme from agricultural waste.

Introduction

Enzymes are important products obtained for human needs through microbial sources. Large number of industrial processes in the areas of industrial, environmental and food biotechnology use enzymes during production (REHMAN and ELAHI 2018). Example of such enzyme are cellulase enzyme which hydrolyze cellulose to simple sugar. These enzymes are mainly produced by microorganisms (TECHAPARIN et al. 2017).

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Cellulases are used in the textile industry, pulp and paper industry, in the production of detergents, for improving digestibility of animal feeds, in food industry and cellulase enzyme account for a significant share of the world enzyme market (BAEZA et al. 2016). The growing concerns about decrease in petroleum supplies and increase in environmental pollution from emission of greenhouse gases and air pollution by incomplete combustion of fossil fuel has resulted in an increased focus on production of bioethanol from lignocellulosics and the possibility of using cellulases to perform enzymatic hydrolysis of the lignocellulosic material (THONGEKKAEW and KONGSANTHIA 2016).

Different microorganisms had been identified as cellulase producers; only a few yeast strains have been seen as producers of cellulase enzyme. For this reason, the search of new strains of yeast from nature is a method for discovering new enzymes which may permit the production of cellulase enzyme at an industrial scale (SHIL et al. 2014). Recently, yeasts have been developed as host organisms for the production of cellulase enzyme (TECHAPARIN et al. 2017). *Trichosporon laibachii*, *Phodotorula glacialis*, *Tetracladium* spp. and *Mrakia blollopis* are known to use both pentose and hexose sugars (CARRASCO et al. 2016). Cellulase production had been reported in several yeast specie; *Saccharomyces diastaticus, Aureobasidium* pullulans and *Wikerhamomyces* spp. (ADELABU et al. 2018, THONGEKKAEW and KONGSANTHIA 2016).

Grasshopper are common insect in the forest regions of West and Equatorial Africa (ADEMOLU and IDOWU 2011b). It has been reported that that gut sections of *Z. variegatus* harbored various microorganisms; bacteria, mould and yeast (JING et al. 2020). These insects are efficient in converting cellulose present in plant materials into glucose with their highly efficient gut systems which can be considered as natural bioreactors (IRENE 2018). They are polyphagous species capable of consuming most of the plant species in its surroundings (ADEMOLU and IDOWU 2011a), and is reported to consume more than 250 plant species among 71 families (ADEMOLU and IDOWU 2011b). Insects *such as Zonocerus variegatus* rely on microorganisms present in their guts to digest plant materials (JING et al. 2020).

Lignocellulosic materials such as corn straw, wheat straw, sorghum straw, rice straw, and sugarcane baggase are alternative materials for cellulase production. These cellulosic materials are cost effective, environmental friendly, readily available and are renewable (REHMAN and ELAHI 2018).

Immobilization is a technique of confining cells or enzymes on organic, inorganic, or hybrid carriers (ZDARTA et al. 2017). Cell immobilization enables easy separation of products from production medium (TECHAPARIN

et al. 2017). An advantage of cell immobilization is the possibility to reuse the cell and thereby reduce production costs (RODRIGUES et al. 2019). Another advantage is increased in stability of the immobilized cell compared to free cell (MUHAMMAD et al. 2019). Cell immobilization can be accomplished by cell support interaction through adsorption, affinity binding, covalent coupling or by entrapment of the cell (ZDARTA et al. 2017). Immobilization materials should have high mechanical strength, resistance to microbial attack, large surface area, many surface groups promoting interaction with the enzyme and should preferably be cheap to produce.

Natural support materials are matrices used for cross-linking of cells or enzymes; this is one of the varieties of materials used for immobilization. Some of the natural support materials used are *Detarium microcarpum*, (KAREEM et al. 2014), *Mucuna urens* (ADELABU et al. 2019), and Vegetable Sponge (OSHO et al. 2014). *Irvingi gbonensis* produce fruits which are mango-like in nature. Research shows that *Irvingi gabonensis* bears nuts which are rich in fat and protein.

Materials and Methods

Insect and gut fluid collection

Adult variegated grasshopper, *Zonocerus variegatus* were collected from a farm at Osiele (latitude 70 10−59 N and longtitude 30 27”0” E) in Odeda Local Governement area of Ogun State, Nigeria. They were collected in clean plastic containers and transported to the laboratory immediately. Diluted bleach (1%) was used for surface sterilization of the insect. This was carried for 30 seconds and rinsed for 1 min in three successive baths. This is to ensure that the gut contents were not contaminated by the surface microflora of the body during dissection (IRENE 2018). The insects were fixed on to a disinfected dissection board and the intestine were exposed from the ventral side with the aid of sterile dissection scissors and fine-tipped forceps to expose the gut (ROJAS-JIMÉNEZ and HERNÁNDEZ 2015). Guts were then transferred into a sterile 1.5 mL Eppendorf tube which contains 0.5 mL of 0.7% (v/v) sterile saline solution, crushed with a pipette tip and all solution (including gut pieces) were used for isolation (SHIL et al. 2014).

Isolation and characterization of yeast

Yeasts present in the gut were isolated by serial dilution of macerated guts of the grasshopper using 1 mL of the preparation. Modified Yeast Extract Peptone Dextrose-Carboxyl Methyl Cellulose (YEPD-CMC) agar containing Yeast Extract; 5 g, Peptone; 10 g, Dextrose; 5 g, NH_4NO_3; 0.2 g, KH_2PO_4; 0.5 g, $CaCl_2.2H_2O$; 0.03 g, $MgSO_4.7H_2O$; 0.03 g, $FeSO_4.7H_2O$; 0.5 g, $MnSO_4.7H_2O$; 0.16 g, $ZnSO_4.7H_2O$; 0.14 g, 1% CMC and Agar; 20 g was used for isolation of yeasts (CARRASCO et al. 2016). Plates were incubated at 30°C for 72 h. Characterization of yeast isolates was carried out based on size, shape and colour. Cell morphology of the purified yeast was studied. Fermentation test using different sugars were also carried out for classification (BARNETT et al. 2000).

Screening of yeast for cellulase production

Qualitative screening

The yeasts were screened for their ability to grow on YEPDA containing Carboxyl Methyl Cellulose (CMC). YEPDA-CMC plates were inoculated with yeast isolates at 30°C for 3 days. Agar plates were flooded with congo red and allowed to stand for 15 min at room temperature, it was de-stained with 1 M NaCl solution for 15 min. The plates that showed zone of clearance around the line of growth indicated cellulose hydrolysis (AMAEZE et al. 2015).

Quantitative screening

Yeasts were assessed for their ability to grow and produce cellulase enzyme. Screening was carried out in Erlenmeyer 250 mL flask containing 100 mL 1% CMC, NH_4NO_3, 0.2 g; KH_2PO_4, 0.5 g; $CaCl_2.2H_2O$, 0.03 g; $MgSO_4.7H_2O$, 0.03 g; $FeSO_4.7H_2O$, 0.5 g; $MnSO_4.H_2O$, 0.16 g; $ZnSO_4.7H_2O$, 0.14 g; Tween-80, 0.1 g. The flasks were inoculated with yeasts and incubated in a rotary (200 rpm) for 120 h at 30°C (AMAEZE et al. 2015).

Selection of starter

Yeast with the best cellulolytic potential was selected, immobilized and used for submerged fermentation of rice husk and saw dust.

Collection and preparation of substrates

Rice husk and sawdust were used for enzyme production. Rice husk was collected from a farm site while sawdust was collected from wood processing industry both in Abeokuta in Ogun State, Nigeria. The substrates collected were oven dried in electric oven at 65ºC and made to powder by grinding in an electric grinder and sieved through a mesh of 4 mm pore size. *Irvingia gabonensis* was obtained from a market in Abeokuta, Ogun State, Nigeria. The seeds of *Irvingia gabonensis* were blended into powder, defatted using Soxhlet extractor and dried in a hot air oven (Oso et al. 2014).

Fermentation of substrates by the yeast

Fermentation of rice husk and saw dust by free yeast was carried out in separate Erlenmeryer flask (250 mL) which contained 100 mL fermentation medium containing NH_4NO_3, 0.2 g; KH_2PO_4, 0.5 g; $CaCl_2.2H_2O$, 0.03 g; $MgSO_4.7H_2O$, 0.03 g; $FeSO_4.7H_2O$, 0.5 g; $MnSO_4.H_2O$, 0.16 g; $ZnSO_4.7H_2O$, 0.14 g; Tween-80, 0.1 g, 10% rice husk and saw dust. Each flask was inoculated with 5% yeast. The fermentation medium was incubated at 30ºC for 120 h. Cellulase activity was measured every 24 h (Carrasco et al. 2016)

Extraction of Enzyme

Crude enzyme was recovered by taking 5 mL from the fermentation medium and centrifuged at 10000 rpm with 7 cm radius, with centrifugal force of 7,826x g for 10 min in a table top high speed centrifuge (HI850R) and the supernatant were taken as the crude enzyme (Thongekkaew and Kongsanthia 2016).

Enzyme Assay

Cellulase assay was carried out using a modified method of Mandels. 0.5 mL CMC (1%) in 0.2 M phosphate buffer (pH 5.0) and 0.5 mL crude enzyme in test tubes. The reaction mixture was incubated at 50°C for 30 min and the reaction was terminated by adding 1 mL of 3,5-dinitrosalicylic acid (DNSA) reagent. The tubes were heated at 100°C in boiling water bath for 5min and then cooled at room temperature. Absorbance was read at 540 nm using a spectrophotometer. Glucose standard was prepared with varying concentration of glucose ranging from 0.1 to 2.0 mg mL^{-1} and treated the same way. Absorbance was plotted against concentration of

glucose to obtain a calibration curve (THONGEKKAEW and KONGSANTHIA 2016). The amount of reducing sugar produced by the action of crude enzyme was read off from the curve. One unit of cellulase activity (U) was defined as the amount of enzyme that liberated 1.0 μmole of D-glucose from substrates (CMC) in 1.0 μL reaction mixture under the assay conditions.

Immobilization of yeast

Immobilization of yeast was carried out with *Irvingia gabonensis* Glutaraldehyde (2.5%) v/v was used to cross-linked 5 g of *Irvingia gabonensis* and the mixture was stirred for 10 min. Yeast suspension was mixed with the slurry obtained and the slurry was used to form spherical beads using a syringe. The slurry was dropped into ethanolic formaldehyde (50 : 50 v/v) for 24 h (KAREEM et al. 2014).

Optimization studies of cellulase production by immobilized yeast cell

Cellulase production from the immobilized yeast was carried out using Mineral Salt Medium which consist of KH_2PO_4 0.8 g, $MgSO_4.7H_2O$ 0.3 g; NH_4NO_3 1.2 g; $FeSO_4.7H_2O$ 0.4 g; $MnSO_4.H_2O$ 1.5 g; $CaCl_2.2H_2O$ 0.3 g; $ZnSO_4.7H_2O$ 1.3 g; Tween-80 0.15 g; peptone 0.75 g, yeast extract 0.3 g; glucose 5 g and 10% each of rice husk and saw dust. The media were incubated at 30°C for 72 h. Samples were taken and analyzed after 72 h of fermentation (KAREEM et al. 2014). Optimization of cellulase production from immobilized yeast cell was carried out considering different parameters; bead size, bead number, *Irvingia gabonensis* concentration, inoculum load and bead reusability. Cellulase production from free and immobilized yeast was also compared.

Statistical analysis

All the experiments were performed in triplicates, results were presented as mean ± standard deviation and were also analyzed by ANOVA using statistical software SPSS version 17.0.

Results

Isolation of yeasts

A total of seven (7) yeasts were isolated from the gut of *Zonocerus variegatus*. Sugar fermentation test showed that all the isolates ferment glu-

cose and sucrose while only one isolate ferment urease and lactose. Biochemical and physiological characteristics of the yeasts identified them as *Trichosporon beemeri, Saccharomyces cerevisiae* 1, *S. cerevisiae* 2, *Candida shehatea, Candida tropicalis, Candida krusei* and *Debaryomyces hansenii.*

Screening for cellulase enzyme

Screening of yeasts revealed that the yeasts hydrolyze cellulose, showing zones of clearance on CMC agar plates. The yeast isolates produced zones of clearance from 22.0 to 35.0 mm in diameter. The highest halo zone was observed with *Candida tropicalis* (35.0 mm) while *Saccharomyces cerevisiae* showed the least halo zone (22.0 mm) (Data not shown). Table 1 showed that the tested yeasts grew and produced cellulase enzyme in mineral medium where CMC was the carbon source. Among the yeasts, *Candida tropicalis* had the highest activity (174.67 U/ml), followed by *Candida shehatae and Debaryomyces hansenii,* which had 160 U/ml. *T. beemeri* had the lowest cellulase activity (94.86 U/ml). Statistical analysis showed that cellulase activity by the yeasts were significantly different ($p \geq 0.05$). *Candida tropicalis* was selected for cellulase production in submerged fermentation.

Table 1

Screening for cellulase enzyme

Yeast isolates	Cellulase activity [µ/mL]
Candida tropicalis	174.67±23.21^{d}
C. shehatae	160.66±10.34^{c}
C. krusei	143.81±11.76b^{c}
Debaryomyces hansenii	160.43±31.68^{c}
Saccharomyces cerevisiae	127.02±13.28^{b}
S. cerevisiae 2	105.86±20.62^{a}
Trichosporon beemeri	94.86±19.17^{a}

Each value is a mean of 3 readings ± standard deviation. Values in the same column followed by the same letter (or subscripts) are not significantly different ($p \leq 0.05$) according to Duncan's Multiple Range Test

Cellulase production from rice husk and saw dust

Cellulase production from rice husk and saw dust by immobilized *C. tropicalis* is shown in Figure 1. The figure showed that cellulase activity increased from 24 hours to 72 hours of fermentation for both rice husk and saw dust; highest cellulase activity was achieved at 72 hour with the

immobilized yeast (234 U/ml) and thereafter decreased for both substrates (Fig. 1). Sharp decrease in cellulase activity was observed in fermented rice husk from 72 hour (198 U/ml) to 96 hour (142 U/ml) – Figure 1. Fermentation of substrates with immobilized *C. tropicalis* showed that saw dust produced the highest cellulase activity (234 U/ml), while rice husk had 198 U/ml cellulase activity (Fig. 1).

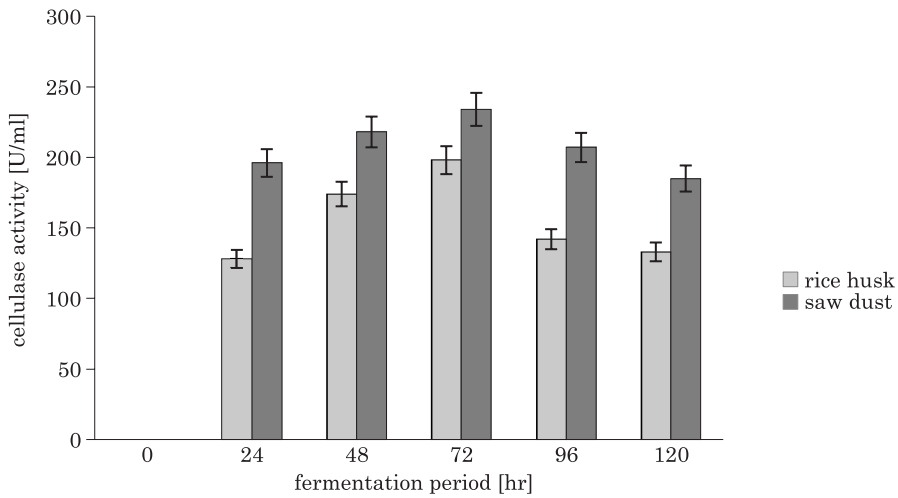

Fig. 1. Effect of fermentation period on cellulase production

Effect of bead size on cellulase production

Figure 2 shows that bead size had significant effect on cellulase production from rice husk and saw dust. Cellulase activity increased with bead size 3 mm to 4 mm and decreased with further increase in bead size. Use of 4 mm bead size produced the highest cellulase enzyme (218.49 U/ml) while fermentation with immobilized *C. tropicalis* of bead size 6 mm had the least cellulase activity (108 U/ml) for both substrates (Fig. 2). Saw dust medium fermented with *Irvingia gabonensis* immobilized *C. tropicalis* showed highest cellulase activity (229.49 U/ml) while rice husk medium had 113.74 U/ml cellulase activity (Fig. 2).

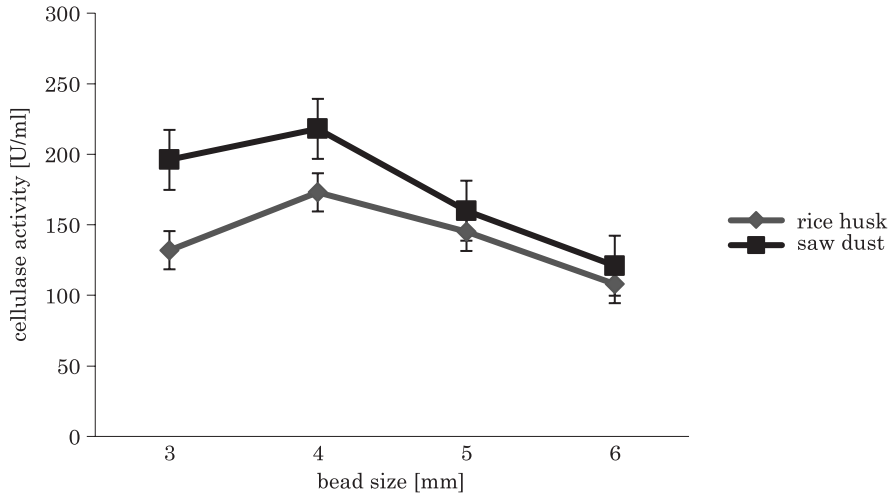

Fig. 2. Effect of bead size on cellulase production

Effect of bead number on cellulase production

Cellulase production from fermentation of saw dust and rice husk by different bead sizes of immobilized *C. tropicalis* is shown in Figure 3. Data illustrated showed that cellulase increased gradually with lower bead number and optimum production was achieved with 6 beads (268.21 U/ml) while fermentation with 10 beads had least cellulase activity (190.47 U/ml) for both substrates (Fig. 3). Maximum production of cellulase enzyme was achieved with fermentation of rice husk by immobilized *C. tropicalis* (268.21 U/ml).

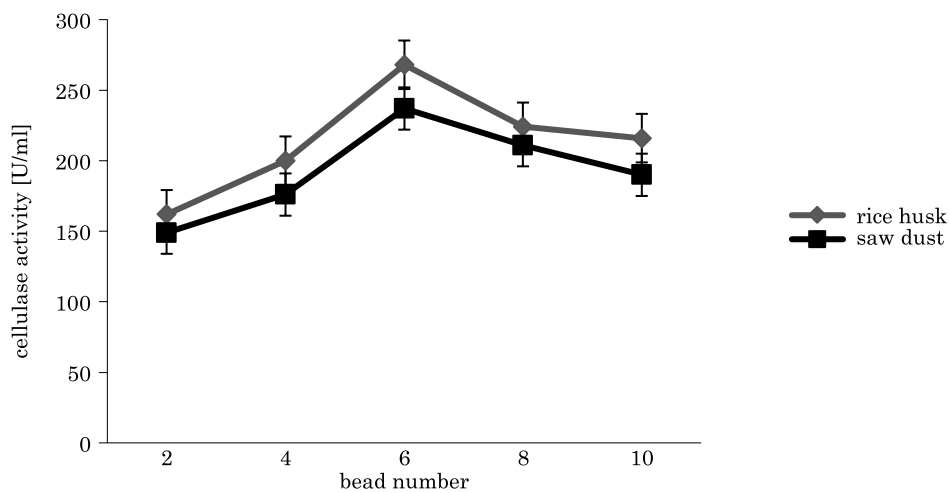

Fig. 3. Effect of bead number on cellulase production

Fermentation of saw dust by immobilized *C. tropicalis* had 237.90 U/ml as its highest cellulase activity and 190.63 U/ml as its least cellulase activity (Fig. 3).

Effect of *Irvingia gabonensis* concentration on cellulase production

Effect of different concentration of *Irvingia gabonensis* on cellulase production was carried out using concentration which ranges from 2–10%. Result presented in Figure 4 showed that fermentation of rice husk and saw dust with immobilized *C. tropicalis* of 6% gel concentration had the highest cellulase activity (246 U/ml) while a decrease in enzyme activity was observed at higher gel concentration (Fig. 4). It was observed from Figure 4 that rice husk fermented with 6% immobilized *C. tropicalis* had the highest cellulase activity (264 U/ml) and (172 U/ml) as its least cellulase activity with 10% *Irvingia gabonensis* concentration. Fermentation of saw dust also had its highest cellulase activity (206 U/ml) with 6% gel concentration while its least activity was 141 U/ml at 10% concentration (Fig. 4).

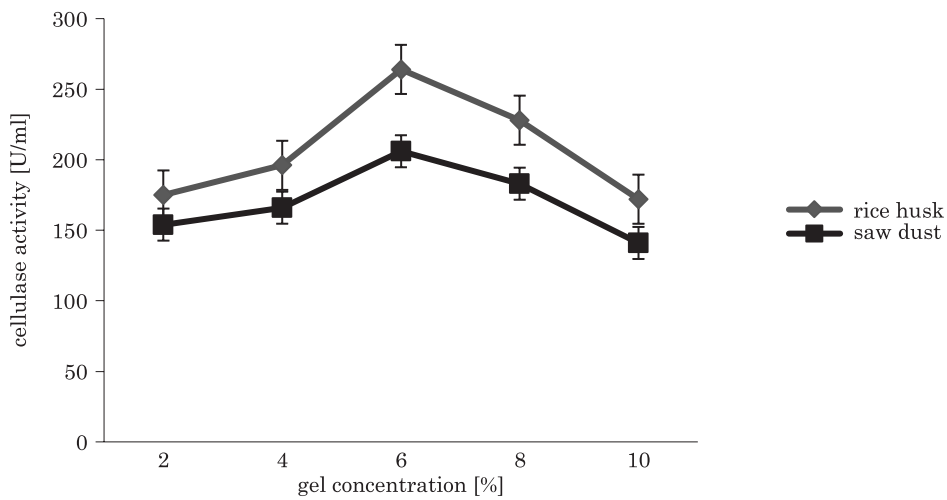

Fig. 4. Effect of *Irvingia gabonensis* concentration on cellulase production

Effect of inoculum concentration on cellulase production

Effect of inoculum load showed that cell load had a very strong influence on cellulase production. Immobilized *C. tropicalis* had maximum enzyme activity at inoculum load of 8% v/v (Fig. 5). Highest cellulase activity observed with use of 8% v/v inoculum in saw dust and rice husk medium

was 172.08 U/ml and 206.5 U/ml respectively. However, higher concentration of yeast did not lead to improved cellulase activity (Fig. 5). Least cellulase activity of 105.03 and 139.01 U/ml was observed for saw dust and rice husk respectively with the use of 10% inoculum concentration.

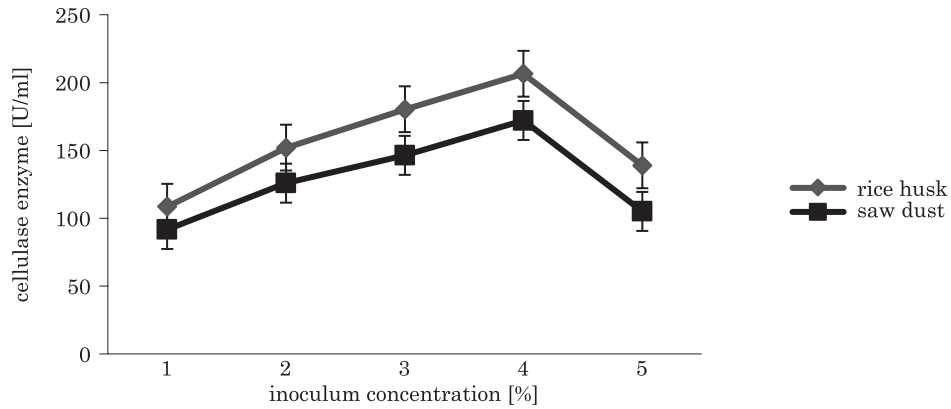

Fig. 5. Effect of inoculum concentration on cellulase production

Effect of reused cross-linked *C. tropicalis* on cellulase production

Potential use of *I. gabonensis* – immobilized *C. tropicalis* on saw dust and rice husk in different cycles during cellulase production was described in Table 2. The results in Table 2 showed that reusing the entrapped cells result in an increase in the enzyme activity during cell re-use for both substrates. Rice husk retained 91, 84 and 75% cellulase enzyme yield for second, third and fourth cycle while saw dust retained 86, 72 and 61% cellulase enzyme yield. Further re-use of yeast cells during the 5th cycle showed a sharp decrease in cellulase enzyme yield (Table 2).

Table 2

Reusability of immobilized *C. tropicalis* on cellulase production

Number of cycle	Cellulase enzyme yield [%]	
	rice husk	saw dust
1	91	86
2	84	72
3	75	61
4	63	58
5	35	20

Each value is a mean of 3 readings ± standard deviation

Comparative cellulase production by free and immobilized cells of *C. tropicalis*

Cellulase enzyme production by free and immobilized cells of *C. tropicalis* was shown in Table 3. Higher cellulase activity was observed in immobilized cells compared to free cells for both substrates (Table 3). The table shows that fermentation with immobilized cells of *C. tropicalis* had higher cellulase activity (132.11 U/ml) compared to its free cells (128.64 U/ml). Fermentation of saw dust with immobilized cells of *C. tropicalis* also had higher cellulase activity (125.31 U/ml) compared to fermentation with free cells (122.91 U/ml) – Table 3.

Table 3

Comparative cellulase production by free and immobilized cells of *C. tropicalis*

Fermentation period	Immobilized yeast		Free yeast	
	rice husk	saw dust	rice husk	saw dust
0	0.00±0.000^a	0 0.00±0.000^a	0.00±0.000^a	0.00±0.000^a
24	124.58±1.192^b	24 118.71±1.126^b	119.64±1.124^b	114.00±1.083^b
48	127.21±1.141^c	121.48±1.121^c	123.35±1.231^c	119.45±1.254^c
72	132.11±1.254^e	126.35±1.213^e	128.64±1.212^e	122.91±1.225^e
96	128.00±1.054^d	122.94±0.697^d	125.11±1.184^d	120.13±1.213^d

Note: each value is a mean of 3 readings ± standard deviation.Various superscripts in Table 3 indicate significant differences ($p < 0.05$)

Discussion

Immobilization of cells for the production of industrially important enzymes offers various advantages such as reduced cost of production, ease separation of cell from production medium, ability to reuse cell in different cycles and reduced possibility of contamination. The immobilization matrix – *Irvingia gabonensis* was noted to provide stability and strength (both physical and mechanical) to this yeast immobilization system because there is no physical change in the beads during cellulase production. This is an advantage over the use of synthetic matrices where disintegration of immobilizing materials was observed (BRETHAUER and WYMAN 2010).

It has been reported that insects are in a set of diffuse mutualisms with yeasts (MADDEN et al. 2018). From the study, *Candida* species were the most frequent yeast isolated from the gut of *Zonocerus variegatus*, this yeast are known as thermotolerant yeast and are able to survive in environment with high pH (KAUR et al. 2018). This had earlier been reported by ADELABU et al (2018) where *Candida* species were isolated from com-

post piles. *Saccharomyces* species had also been isolated from the gut of black beetle. KAUR et al. (2018), they have been reported to utilize wide range of nutrients and can secret different enzymes (PADILLA 2016). *Debaryomyces hansenii* and *Trichospron beemeri* had been reported by different authors as yeasts isolated from insect gut (ADELABU et al. 2018).

Insects have developed very effective strategies to use lignocellulosic materials as source of energy (WILLIS et al. 2010). *Zonocerus variegatus* had been reported to established symbiotic interaction with different yeasts to carry out key hydrolytic activities (ADEMOLU and IDOWU 2010a). Screening of the yeast isolates confirmed that the yeasts isolated from gut of *Zonocerus variegatus* were able to utilize Carboxyl Methyl Cellulose (CMC), but their ability to degrade CMC differs. Differences in their ability to hydrolyze the substrates could be due to the amount of enzyme excreted into the medium and different growth rates of the yeast strains (PINJARI and KOTARI 2018).

The yeasts were confirmed as cellulose degrading yeast because they all grew on 1% CMC, this is an indication that the yeasts are potential cellulase producers. *Candida tropicalis* had the highest cellulase activity. This specie of *Candida* might possess more exogluconases, endogluconases, and β-glucosidases which are classes of cellulase enzyme than other species isolated in this study. This is confirmed by THONGEKKAEW et al. (2019) who reported that *Candida* species isolated from gut of insect assimilated D-xylose. Least cellulase activity was observed with *Trichospron beemeri,* this yeast might have low xylose reductase (XR) and xylose dehydrogenase (XDH) which are enzymes responsible for xylose and cellulose degradation (KHAN and DWIVEDI 2013). Though the yeast are of the same general, the gene that code for cellulase production in each of them might differs, hence the difference in the amount of cellulase enzyme produced.

Fermentation time is an important factor from an economic point of view in enzyme production. The findings on the effect of fermentation time on enzyme production from rice husk and saw dust revealed that *Candida tropicalis* produced cellulase from both agrowastes. Enzyme production increased with increase in fermentation time with each of the agrowastes, such increase may be due to the gradual breaking down of complex sugars to simple sugar during fermentation (REHMAN and ELAHI 2018). Saw dust fermented with *C. tropicalis* was shown to be the best, yielding a higher amount of cellulase enzyme. When comparing cellulase production in this study to earlier reports, activities of *C. tropicalis* was higher than that of ZDARTA et al. (2017) who reported 164 U/ml as the highest cellulase production from compost piles.

Sizes of beads determine the number of cells available for the production of cellulase enzyme. Optimum cellulase production was achieved with bead size of 4 mm, which indicate that at this size, the number of pore spaces made available is highest and the number of biomass occupying each space is maximum (XING et al. 2015). Smaller bead size exhibited better cellulase production, when compared with large-size beads. This might be due to increased sur-face area of the bead, which enhances the mass transfer.Thus smaller beads (4 mm) have more surface area per unit volume and hence more productivity (AHMED et al. 2019).

It was observed that maximum cellulase production was obtained with 6 beads. Enzyme production decreased at high initial cell loading (10 beads/flask). This could be attributed to the fact that, when the number of beads increases, the nutrient/bead ratio decreases, which may become limiting (DEVI and NAGAMANI 2018). Also high cells loading had been observed to result in low yield, because cells entrapped in *Irvingia gabonensis* may experience diffusional problem due to inability of the immobilized cells to access nutrients in the fermentation broth (RODRIGUES et al. 2019).

Reports had shown that concentration of immobilized material can enhance enzyme production (DEVI and NAGAMANI 2018, DONG et al. 2013, KAREEM et al. 2014). The effect of different gel concentration showed that cellulase enzyme yield was optimum at gel concentration of 6% while a decrease in cellulase enzyme activity at higher gel concentration, this may be due to diffusional limitations imposed by the solid nature of the hardened matrix (OSHO et al. 2014). Increasing in bead concentration did not cause any improvement in cellulase production, but caused some leakage problems. Also, increasing the beads concentrations caused reductions in enzyme production because the beads settled at the bottom of the fermentation medium (FERNANDEZ-LAFUENTE 2019).

Studies on inoculum concentration showed that highest cellulase activity was observed in *C. tropicalis* fermented rice husk. Lower cellulase biosynthesis at lower inoculum size was probably due to lower cell density in the bead at low cell loadings (MAJOLAGBE et al. 2010). The positive effect of increasing cell loading from 3 to 4%, which led to improve cellulase production, was the same results obtained for the production of gluconic acid by *Aspergillus niger* immobilized in Calcium alginate beads (DONG et al. 2013). Decreased yield at higher inoculum size is probably due to nutritional imbalance caused by tremendous growth resulting in autolysis of cells (DEVI and NAGAMANI 2018). This decrease in enzyme production with further increase in inoculum might also be due to clumping of cells which could have reduced sugar and oxygen uptake rate and also, enzyme release

(MUHAMMAD et al. 2019, SIKANDER et al. 2017). Similar observation was also reported by KOURKOUTAS et al. (2004) where maximum ethanol yield was obtained at 4% inoculum size.

During repeated use of immobilized *C. tropicalis* cells, it was observed that increase in cellulase production in the first cycle was considerably lower than those observed in second and third cycles. This might be due to the fact that during the first cycle, cells are in an adaptation phase and they might be suffering stress caused by immobilization (DEVI and NAGAMANI 2018). Decrease in cellulase enzyme was also observed as the re-use number increased. This may be as a result of clogging of yeast cells immobilized within the matrix. IKEDA et al. (2015) reported that accumulation of enzyme inside the cell inhibited activity of endo-glucanases, exo-glucanases and β-glucosidases found in cellulase-system and thus led to decrease cellulase enzyme production.

Comparative cellulase enzyme production by free and immobilized cells of *C. tropicalis* showed that Immobilized cells gave an improved cellulase enzyme yield. This result agreed with KAREEM et al. (2014) who produced citric acid from free and immobilized cells. BAYRAKTAR and MEHMETOGLU (2012) reported that immobilized cells offer several advantages over free cells such as decreased medium viscosity, enhanced oxygen and nutrient transfer, higher productivity, operational stability. Use of immobilized cells also prevent decreased contamination of the product by free cells.

Reports has shown that other methods of immobilization are entrapment, crosslinking encapsulation and covalent bonding. Covalent bonding is widely used for immobilization. This involves formation of covalent bonds between the chemical groups in enzyme and the chemical groups on the support or carrier. Each immobilization technique has its advantages and disadvantages.

Conclusion

Result in this study showed that saw dust and rice husk have desirable properties for cellulase production. Immobilized *C. tropicalis* showed higher cellulase activity compared to its free cells, thus immobilized yeast can be used for cellulase production from cost effective materials.

Accepted for print 17.07.2022

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POLISH JOURNAL OF NATURAL SCIENCES
Abbrev.: Pol. J. Natur. Sc., Vol 37(2): 205–218, Y. 2022

DOI: 10.31648/pjns.7819

PROTECTIVE EFFECT OF HONEY AGAINST ALUMINIUM-INDUCED ERYTHROCYTE OSMOTIC FRAGILITY AND HEMOGLOBIN DENATURATION*

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Key words: honey, ascorbic acid, aluminium, human erythrocytes, hemoglobin.

Abstract

The aim of the present study was to investigate the effects of honey against aluminium (Al) and hypotonic pressure-induced hemolysis of human red blood cells (RBCs) and hemoglobin degradation *in vitro*. Human RBCs were pretreated separately with honey samples and ascorbic acid. Four different treatment groups were considered: untreated RBCs (negative control), aluminium treated RBCs (positive control), honey + aluminium treated RBCs, and ascorbic acid + aluminium treated RBCs. Samples were then evaluated by simultaneous measurement of cellular turbidity and hemoglobin (Hb). The results showed that RBCs suspensions treated with honey samples, particularly honey 2 presented highest cell and Hb values compared to that of ascorbic acid and positive control, whereas, ascorbic acid exhibited a prooxidant effect on cell and Hb. The results of this study suggest that honey not only protect cell integrity but also prevent oxidative degradation of Hb. Therefore, the present study demonstrated a protective effect of honey against Al-induced red blood cells hemolysis and hemoglobin degradation.

Introduction

Aluminium (Al) is the third most abundant element found in the earth's crust (GUPTA et al. 2013). It has become integral part of our modern life and can be found in food (corn, yellow cheese, salt, herbs, spices, tea), water, cosmetics, medicines such as vaccines, deodorants, transport vehicles and electronic appliances (NIU 2018). Al is considered as a non-essential trace element of low toxicity in human beings (SINGLA and DHA-

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* This work was funded by the Algerian Ministry of Higher Education and Scientific Research (PRFU project grant no D01N01UN340120180001).

WAN, 2013), and occurs naturally in the air, water and soil, and across to the body via the respiratory system, skin or gastrointestinal tract which can reach and accumulate in almost all mammalian tissues, including brain, liver, kidneys, heart, blood and bones (JAISHANKAR et al. 2014). The accumulation of Al in tissues and organs leads to their toxicity and dysfunction (VERSTRAETEN et al. 2008, RAHIMZADEH et al. 2022). Several diseases such as dialysis dementia, Alzheimer's disease, Parkinson's disease, osteomalacia and microcytic anemia have been associated with elevated levels of Al (JEFFERY et al. 1996, BONDY 2010, GOULLE and GRANGEOT-KEROS 2020).

Al causes numerous changes in peripheral blood and hemogenic system (OSIŃSKA et al. 2004). It induces hypertension and disturbs the function of erythrocyte membrane (ZHANG et al. 2016). Al bound to transferrin in blood, easily penetrates the erythrocytes, causes alteration of erythrocytes membrane and decreases osmotic resistance (OSIŃSKA et al. 2004). In addition, Al has been reported to generate reactive oxygen species, which causes peroxidative damage to lipids and proteins membrane (SAEED et al. 2021).

In recent decades, much interest has been generated by scientists and epidemiologists for wide ranges of natural antioxidants from food, particularly fruits and vegetables with reports demonstrating their protective effects against a growing list of aging diseases (WIART 2013, LIGUORI et al. 2018). Honey is one of the most used natural products by humankind for the treatment of diseases. It has a great variety of pharmacological activities, particularly antioxidant, antimicrobial and antiviral activity (BERETTA et al. 2005, ISRAILI 2014, BELLIK and SELLES 2017), treatment of wounds, burns (TASHKANDI 2021), skin ulcers (MCLOONE al. 2020) and inflammations (EL SEEDI et al. 2022). The healing property of honey is due to its chemical composition (BERETTA et al. 2005). Honey contains various amounts of polyphenols and flavonoids which confer it good antioxidant properties. The antimicrobial action is due to its acidity, hydrogen peroxide content, osmotic effects, nutritional and antioxidant content (ALZAHRANI et al. 2012).

There are several scientific reports indicating a great variety of pathological effects of Al, however, very little experimental research works have been conducted on the protective effects of natural antioxidants on Al-induced oxidative damage in human red blood cells. RBCs are particularly prone to oxidation because of their high polyunsaturated lipids content, exposure to oxygen and the presence of transition metals, such as iron and copper (KUHN et al. 2017). Furthermore, Hb inside and outside the RBCs undergoes constant transformation from oxyhemoglobin (Fe^{2+}-Hb) to met-

hemoglobin (Fe^{3+}-Hb). This metHb may cause its own oxidation (autooxidation) or be oxidized by other agents, and so lose its capacity to carry oxygen and become highly reactive and disruptive agent (ALAYASH 2022). Thus, RBCs are considered as a useful model *in vitro* for studying oxidative stress, elucidating the mechanisms involved in metHb reduction (KINOSHITA et al. 2007), and investigating the antioxidant potency of foods (PAIVA-MARTINS et al. 2009). The goal of the current study was to evaluate the protective role of honey on RBCs osmotic fragility and Hb degradation under Al-induced oxidative damage in human erythrocytes.

Methods

Chemicals

Folin-Ciocalteu, anhydrous sodium carbonate, aluminium chloride, gallic acid, quercetin, ascorbic acid, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, sodium chloride, formalin, were purchased from Sigma Aldrich (Germany) and Biochem Chemopharma (France).

Honey samples

Two multi-flora honey samples (25 ml for each sample) were used in this study. Honey samples were collected from beekeepers in March 2018 in the regions of Bir Snab and Colla of the city of Bordj Bou Arreridj, located in Northeastern Algeria (latitude 36.3479 and longitude 6.650773). The regional climate is semi-arid, T_{max} ranges between 32 and 37°C, while the T_{min} varies between 0 and 5°C. Average annual rainfall varies between 200 and 500 mm (KOURAT et al. 2022). Honey samples were given numbers prior experiment (Honey 1 for sample collected form Bir Snab and Honey 2 for sample collected from Colla) and were stored in a refrigerator in airtight plastic containers until analysis.

Preparation of honey extracts

During the sample preparation, 1 g of each pure honey was diluted with phosphate buffered saline and was made up to 10 mL followed by a through mixing. The final concentration of each honey extract was 100 mg/mL (BERETTA et al. 2005).

Total phenolic content

The total phenolic content in each honey sample was estimated using Folin-Ciocalteu method (SINGLETON and ROSSI 1965). Briefly, aliquots (200 µL) of honey extract or standard solution of gallic acid (0.02, 0.04, 0.06, 0.08 and 0.1 mg/mL), used to establish the calibration curve, were added to 500 µL of Folin-Ciocalteu reagent (10%). The reaction mixture was thoroughly mixed by shaking. The mixture was incubated at room temperature for 5 min, before 1500 µL of Na_2CO_3 (7.5%) were added. All the reaction mixtures were then shaken and incubated for 30 min. The absorbance of blue mixtures was recorded at 765 nm using a double beam UV-Visible spectrophotometer (Shimadzu UV-1601, Japan). Total phenolic contents were expressed as milligram of gallic acid equivalent (GAE)/g of honey by using an equation obtained from the standard curve of gallic acid: $y = 9.04x + 0.029$ ($R^2 = 0.998$).

Total flavonoid assay

The total flavonoid content in each honey sample was measured by colorimetric assay (JAIN et al. 2011), using quercetin for preparing the calibration curve. One mL of honey extract or standard solution of quercetin (0.005, 0.01, 0.02, 0.03 and 0.04 mg/mL) was reacted with 1 mL of aluminium chloride (2%). After incubation at room temperature for 1 hour, the absorbance of the reaction mixture was measured at 420 nm using a double beam UV-Visible spectrophotometer (Shimadzu UV-1601, Japan). Total flavonoid contents were calculated as milligram of quercetin equivalent (QE)/g from the calibration curve: $y = 6.406x + 0.012$ ($R^2 = 0.993$).

Isolation of human erythrocytes

Peripheral blood was obtained from apparently healthy adult volunteers, with no clinical indications of anemia. This study was conducted in accordance with the principles outlined in the Declaration of Helsinki. For each analysis, 4 mL of blood were centrifuged at 3000 rpm for 10 minutes at 4°C using a laboratory centrifuge (Sigma 2-16KHL – Germany). Platelets, leukocytes and plasma were removed by aspiration. RBCs were washed three times and re-suspended in phosphate buffered saline (PBS: 123 mM/L of $NaH_2PO_42H_2O$, 27 mM/L of Na_2HPO_4; pH 7.4) to a final hematocrit of 35% (vol/vol).

The samples tested were: negative control (50% RBCs suspension: 50% PBS), positive control (50% RBCs suspension: 50% PBS), ascorbic acid (50% RBCs suspension and 50% ascorbic acid [50 mM/mL]), honey 1

(50% RBCs suspension: 50% honey 1 [100 mg/mL]) and honey 2 (50% RBCs suspension: 50% honey 2 [100 mg/mL]). All samples were incubated with 800 µL $AlCl_3$ for 30 minutes, except the negative control which was treated with an isotonic solution (PBS). All samples were evaluated simultaneous for hemoglobin and cellular turbidity. The latter is the indicative of intact cells, as demonstrated previously (TAKEBAYASHI et al. 2012).

Cellular turbidity measurement

After incubation for 30 minutes at 37°C, 100 µL from each sample were collected and 200 µL of hypotonic solution (0.7% of NaCl) were added to induce cellular fragility. The mixture was allowed to stand for 15 minutes at room temperature and 100 µL of formalin (4%) were added to fix the cells. An aliquot (100 µL) of the reaction mixture was diluted 20 times with PBS and the optical density was recorded at 620 nm (Shimadzu Corporation, Japan 1601UV) (BELLIK and IGUER-OUADA 2016).

Hemoglobin measurement

Briefly, RBCs suspensions were diluted 20 times and centrifuged at 3000 rpm for 10 minutes at 4°C. Subsequently, 2 mL of supernatant were recovered and the optical density was measured at 412 nm (BELLIK and IGUER-OUADA 2016).

Microscopic study

To study morphological changes in RBCs by optical microscopy, 10 µL aliquots of RBCs were taken directly after measurement of cellular turbidity. The samples were mounted on a slide with a cover slip and examined under a microscope at 10×100 magnifications and images were recorded.

Hemoglobin stability evaluation

Regarding hemoglobin (Hb) stability test, the same procedure was used as for cell turbidity, except the RBCs suspension was replaced by Hb alone. Erythrocytes suspensions, prepared as described above, were lysed in a 999-fold volume of distilled water. Hb stability was recorded spectrophotometrically at 412 nm over a time period of 24 h. The decrease in absorbance at 412 nm represents the degree of hemoglobin breakdown. Briefly, after incubation for 30 minutes, Hb (1 mL of hemolysate) was treated with 800 µL of $AlCl_3$, except the negative control which was treated with an isotonic solution (PBS) and Hb measured for 24 h (BELLIK and IGUER-OUADA 2015).

Statistical analysis

All experiments were repeated at least three times and the data are shown as means ± S.D. Statistical examination was performed with the analysis of variance (ANOVA) using Statistica Software version 5.5 (Statsoft, France). Values were considered to be significant when P was < 0.05.

Results and Discussion

Total phenolic and flavonoids content of honeys

Polyphenols are an important group of compounds that were reported to influence not only the appearance but also the functional properties of honey (Becerril-Sánchez et al. 2021). A significant difference was observed in the phenolic contents of the studied honeys. Honey 2 contained the highest phenolic content with mean value of 1.55 ± 0.04 mg GAE/g while honey 1 showed a concentration of 0.63 ± 0.03 mg GAE/g. Our results are higher than that reported by Akgün et al. (2021), and Habryka et al. (2020), who determined average levels TPC of 0.26±0.07 mg GAE/g and 0.3 mg GAE/ g in multifloral honey, respectively. As with the phenolic content, honey 2 showed the highest levels of flavonoid content (0.17 ± 0.003 mg QE/g) when compared to honey 1 (0.075 ± 0.005 QE/g). The obtained results are in a quite agreement to the range reported by Socha et al. (2016) (5.26 to 14.39 mg QE/100 g of flavonoids) but much higher than that of Habryka et al. (2020) (2.77 mg QE/100 g). Various studies showed that the multi-floral honeys with the highest phenolic compound contents. This high content also confers a high antioxidant capacity.

Table 1

Total phenolic and flavonoid contents of honey samples

Samples	Phenolic content [mg GAE/g]	Flavonoid content [mg QE/g]
Honey 1	0.63 ± 0.03^{a}	0.075 ± 0.005^{a}
Honey 2	1.55 ± 0.04^{b}	0.17 ± 0.003^{b}

Measurement of erythrocyte and hemoglobin

Figure 1 shows the absorbance values of RBCs control samples (negative control and positive control) and treated samples (ascorbic acid, honey 1 and honey 2). Negative control sample presented the highest values of cells compared to the all other studied samples, the reason is that

negative control sample was not subjected to the oxidation by $AlCl_3$. Suspensions treated with honey samples showed highest cells values compared to that of ascorbic acid and positive control samples.

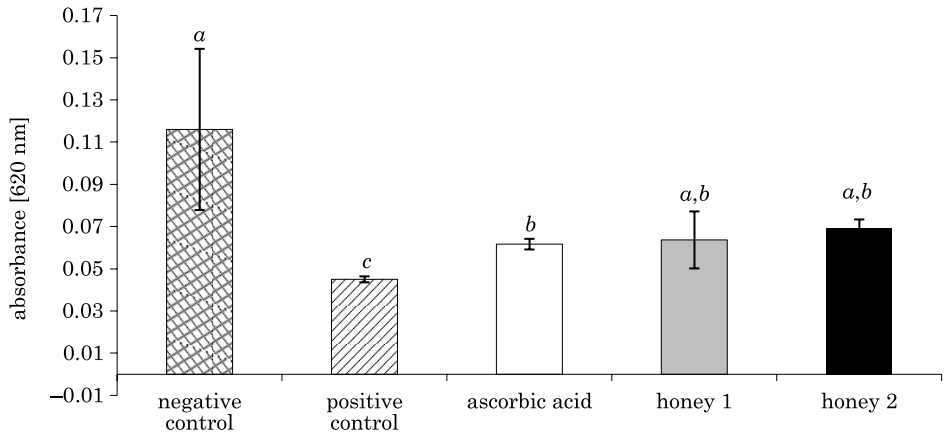

Fig. 1. Cellular turbidity of human erythrocytes when exposed to the hypotonic conditions and oxidation by $AlCl_3$. Values are means ± S.D. of triplicate experiments

Similarly, Hb absorbance was much higher in samples supplemented with honey extracts than in untreated positive control sample (Fig. 2). These seemingly contradicting results suggest honey, particularly honey 2, not only protect cell integrity but also prevent oxidative degradation of Hb.

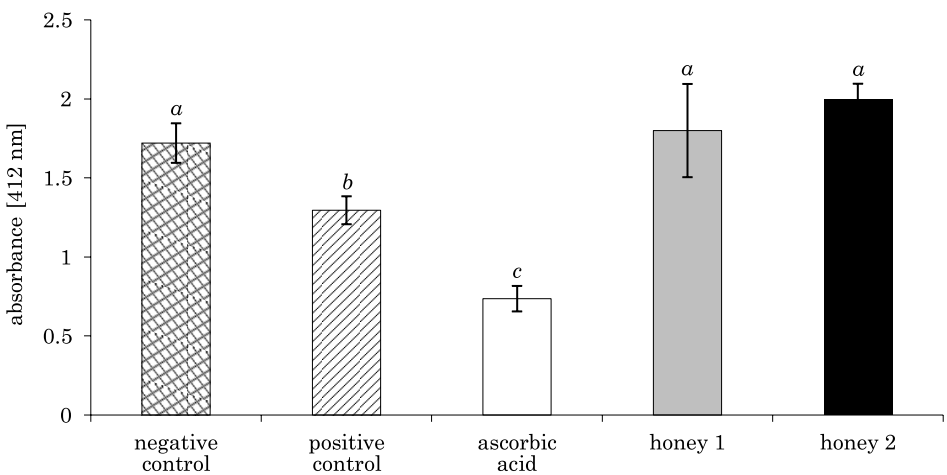

Fig. 2. Hemoglobin levels of human erythrocytes after oxidation by $AlCl_3$. Values are means ± S.D. of triplicate experiments

Morphological analysis of erythrocytes

With respect to cell morphology, the untreated positive control cells (Fig. 3) as well as suspension cells treated with ascorbic acid showed cytoplasmic membranes abnormalities, whereas negative control cells and erythrocytes treated with honey samples displayed normal cell shape (Fig. 3).

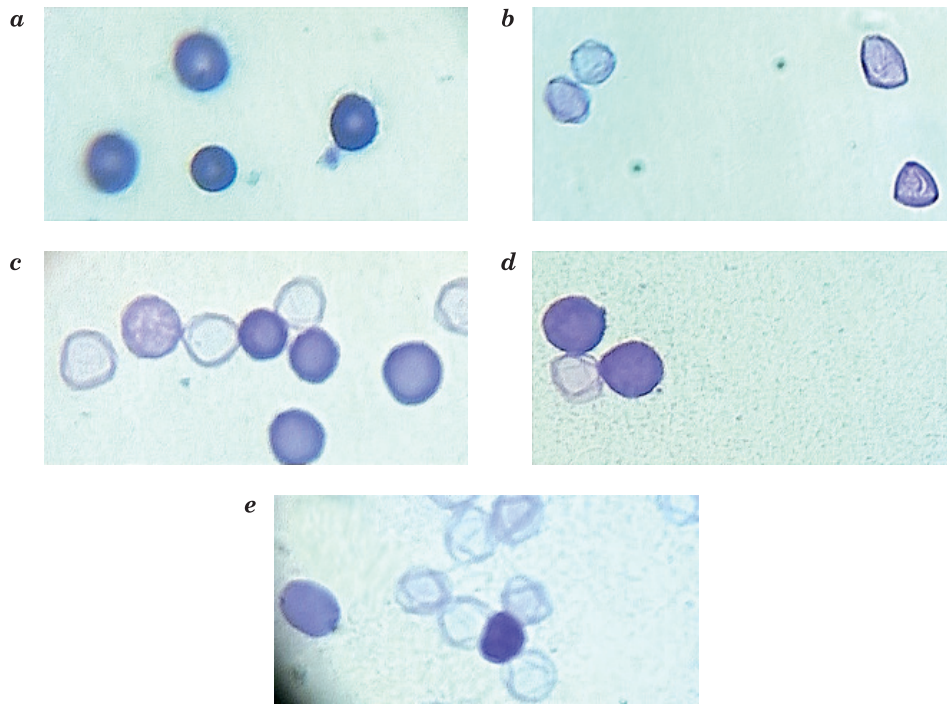

Fig. 3. Microscopic images of erythrocyte morphology under hypotonic conditions and after the completion of oxidation by $AlCl_3$: *a* – negative control; *b* – positive control; *c* – erythrocytes treated with honey 1; *d* – erythrocytes treated with honey 2; *e* – erythrocytes treated with ascorbic acid

Hemoglobin stability measurement

The kinetic measurement of Hb stability in the presence of honey samples and ascorbic acid was also investigated and the results were shown in Figure 4. A statistically significant decrease in Hb absorbance was observed after 30 minutes of incubation. Nevertheless, the decreases varied according to the studied samples. Honey samples were effective in protecting human Hb against oxidative damage when comparing to the positive control. As shown in Fig. 4, Hb absorbance remained significantly higher in samples treated with honey extracts. However, ascorbic acid appears to

accelerate the denaturation of Hb due to the significant reduction in absorbance from 30 minutes of incubation, and persists until the end of the test (Fig. 4).

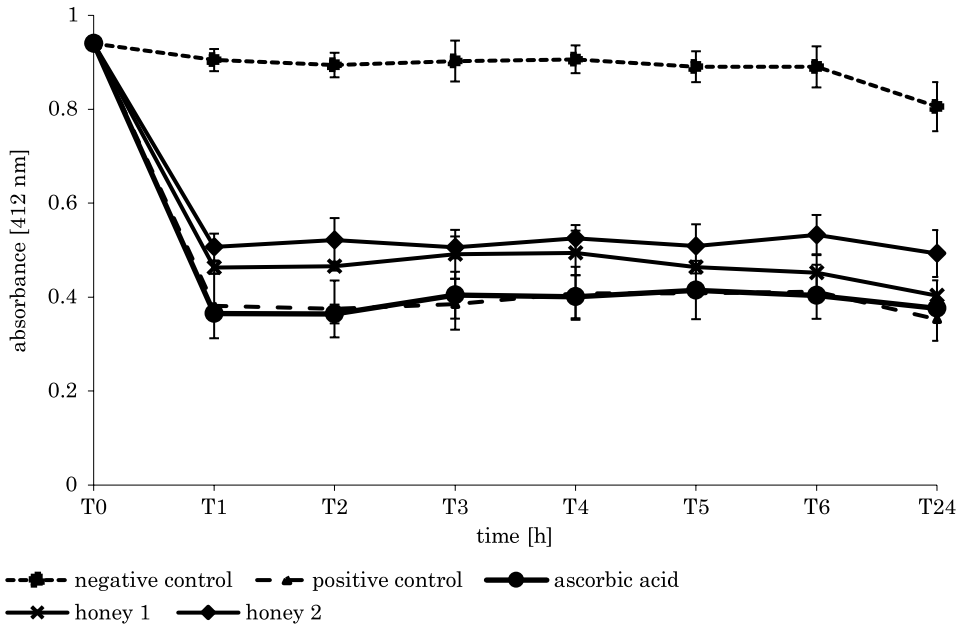

Fig. 4. Kinetics of hemoglobin breakdown exposed to $AlCl_3$

It is worthy noting that negative control sample presented the highest values of Hb compared to the all other studied samples; the reason is that negative control sample was not subjected to the oxidation by $AlCl_3$.

A considerable number of reports suggest that aluminium may present a major threat for humans, animals and plants in causing many diseases (JAISHANKAR et al. 2014, RAHIMZADEH et al. 2022). This critical component can alter membrane phospholipid metabolism either by its direct or indirect interaction and may disturb membrane fluidity and integrity (SINGLA et al. 2013).

In the present study, we demonstrated that honey samples were effective in protecting erythrocytes against hypotonic stress and Al-induced adverse effects with respect to cell absorbance (Fig. 1) and morphology (Fig. 3). This protection against hypotonic stress is most likely due to an increase in membrane fluidity resulting from prior exposure to honey. Several authors outlined the importance of membrane fluidity for the stability of erythrocytes (TSUCHIYA et al. 2002, DUCHNOWICZ et al. 2021). In addition, it is well demonstrated that erythrocytes become more resistant to

hypotonic solution and detergents when membrane fluidity is increased by treatment with tocopherols (SUZUKI et al. 1993). TSUCHIYA et al. (2002) reported that erythrocytes were more resistant to physical stress in conjunction with an increase in membrane fluidity caused by treatment with propofol. Moreover, it has been reported that flavanols and procyanidins interact with membrane phospholipids through hydrogen bonding with the polar groups of phospholipids, these compounds can accumulate at the membranes surface, both inside and the cell, and contribute to maintain membranes integrity by preventing access of deleterious molecules to the hydrophobic region of the membrane (VERSTRAETEN et al. 2015, VILLALAÍN 2022).

The maintenance of the cation gradient by high affinity Ca^{2+}-ATPase is fundamentally important in the control of hydration, volume, nutrient uptake, and fluidity of cells (JEWELL et al. 2013, OLURANTI et al. 2021). Low-affinity Ca^{2+}-ATPase is considered to be responsible for the shape and deformability of the erythrocytes membrane.

Energy production in the form of ATP is necessary for maintaining RBCs function. As these cells lack mitochondria, energy in RBCs is generated only through glycolysis and pentose phosphate pathway. In this study, hemolysis was attenuated in the presence of honey which is a rich source of glucose. The latter is considered as a main substrate for energetic metabolism of erythrocytes (VISKUPICOVA et al. 2015). Whereas, energy depletion is well known to induce and mediate apoptosis (LANG and LANG 2015). This may be the main reason for the observed low values of RBCs in other untreated samples under oxidative conditions (positive control and ascorbic acid samples).

Ascorbic acid alone has no appreciable effect on osmotic fragility and Al-inducing oxidative damage. The reason why ascorbic acid alone failed to protect erythrocytes may also be due to its prooxidant activity (PODMORE et al. 1998, KAZMIERCZAK-BARANSKA et al. 2020).

Recently, there is growing evidences that cell-free Hb is more susceptible for oxidative modifications than intracellular Hb (XIANG 2013, AGYEMANG et al. 2021, ALAYASH 2022). Hb within red blood cells is protected from oxidative processes by enzymatic and antioxidant molecules as well as the RBCs membrane that provides a physical barrier against oxidation. In the other hand, the redox chemistry of the released Hb is susceptible to toxic reactions due to the interaction with oxidative stress agents such as peroxide, yielding free radicals and highly-oxidizing states at the iron (ferryl, Compound II) (VOLLAARD et al. 2005, AGYEMANG et al. 2021). In addition, Hb amount present in the circulation has been related to hemolysis, vascular diseases, and stroke-related intracerebral hemorrhage (WOOLLARD et al. 2009, SCHAER and BUEHLER 2013).

In agreement with previous cell-free Hb studies (XIANG et al. 2013). The decrease in optical density of Hb corresponds to its inactivation. Inactivation and degradation of Hb are two related processes. In the present work, Hb was found at higher levels when samples were treated with honey, prior to treatment with $AlCl_3$, suggesting a protective effect against inactivation and degradation of Hb. HATHAZI and coworkers (2018) found that caffeic acid slowed down significantly the nitrite-induced oxidation of Hb. This is in line with our previous findings that ginger acts to efficiently reduce the oxidation and degradation of Hb (BELLIK and IGUER-OUADA 2015). However, treatment with ascorbic acid did not protect against Hb degradation. It was found that ascorbic acid exhibited a pro-oxidant effect on both cellular membrane and Hb. SIMONI et al. (2009) reported that ascorbic acid without glutathione failed to protect Hb against hydrogen peroxide-mediated oxidation of Hb and formation of its ferryl intermediate. In addition, It has been demonstrated that Hb breakdown rate is correlated to erythrocytes hemolysis in the presence of ascorbic acid (IBRAHIM et al. 2006, BELLIK and IGUER-OUADA 2016).

According to TIMOSHNIKOV et al. (2020), ascorbic acid could react with oxygen to form the hydrogen peroxide, which could be further react with hemoglobin to form MetHb and superoxide, the superoxide generated in the heme pocket can oxidize the tetrapyrole rings, leading to the degradation of heme and the release of iron. Free heme can react with lipids in cellular membranes, inducing lipid peroxidation and, increase cell permeability leading to hemolysis, which may explain the high level of hemolysis observed in erythrocyte suspensions not treated with honey.

Conclusion

This study demonstrated that honey protected erythrocytes from hemolysis and Hb from denaturation. It can be assumed that honey has the potential to reverse the aluminium induced adverse effects on membrane and has an important role in preserving cell integrity. As well, our result corroborates the fact that uptake of natural nutrients including honey, fruits and vegetables is more effective at boosting antioxidant activity than ascorbic acid supplementation.

Conflict of interest

The author declares that there is no conflict of interest.

Accepted for print 5.02.2022

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POLISH JOURNAL OF NATURAL SCIENCES
Abbrev.: Pol. J. Natur. Sc., Vol 37(2): 219–232, Y. 2022

DOI: 10.31648/pjns.7408

DISTRIBUTION, HABITAT USE MAPPING AND CONSERVATION THREATS OF FISHING CAT (*PRIONAILURUS VIVERRINUS*) IN SHUKLAPHANTA NATIONAL PARK, NEPAL

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Key words: habitat use, relative threat ranking, scat, pugmark, transect walk, wetland.

Abstract

The fishing cat (*Prionailurus viverrinus* Bennett, 1833) is a medium sized globally vulnerable wild cat of South and Southeast Asia. The Government of Nepal lead the periodic monitoring of big cats and their prey species, however such monitoring program focusing on small cats including fishing cat has almost lacking in the Western part of Nepal. Considering this gap in knowledge, we used strip transect (1–2 km * 5 m) and key informant survey to understand the current distribution, habitat use and conservation threats to fishing cat in Shuklaphanta National Park (ShNP) of Sudurpaschim Province of Western Nepal. Our findings indicate that the fishing cat was distributed in and around the wetland habitats. Furthermore, the majority of sign records were found in wetland with sparse sal forest, riverine forest and grassland with marshy areas. Using relative threat ranking method, we identified over fishing, wetland depletion and lack of recognition of fishing cat habitat as crucial threats to fishing cat. Restoration of wetland habitats, enhancement of fish densities in the wetlands and conservation awareness programs focusing fishing cat distribution sites should no longer be neglected in conservation planning to ensure their survival. We also recommend the systematic camera trapping and genetic level study of sub-population in ShNP and adjacent areas, as well as habitat use by radio-collaring.

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Introduction

The fishing cat (*Prionailurus viverrinus*) is a medium sized small cat species with stocky, powerful build, short legs and has webbed paws and a short tail to be used as rudder in water for swimming (TIMILSINA et al. 2021). It has elongated face, small ears that are poisoned far back on their head, short legs and a short tail. It is one of those small felines characterized by noticeable strips and spots patterns on the head, face and body (THUDUGALA and RANAWANA 2015). A fully grown fishing cat weights about 5–16 kg (DUGAN 1993). This elusive and moderately distributed aggressive animal is locally known as Malaha biralo (JNAWALI et al. 2011) The fishing cat is a globally threatened small cat species enlisted as "Vulnerable" in the IUCN Red List with a decreasing population range in South and Southeast Asia (MUKHERJEE et al. 2016) and appended in the Appendix II of *Cites* for enhancing conservation initiatives (*Cites*. 2020). Fishing cats have a wide range of patchy distribution throughout their range, primarily in low lands of South and Southeast Asia (MUKHERJEE et al. 2016, MISHRA et al. 2018, SILVA et al. 2020) with strongholds in Sri Lanka, Bangladesh, India and Nepal (MUKHERJEE et al. 2016). Their distribution range is shrinking globally with loss and degradation of wetlands, land degradation due to increasing soil erosion and sedimentation, industrialization, urbanization, and global climate change (CHOWDHURY et al. 2015, MISHRA et al. 2020, MUKHERJEE et al. 2012, TAYLOR et al. 2016). Nationally, fishing cat has been categorized as "Endangered" species with their distribution believed to encompass large parts of the southern lowland of Nepal's Terai region (JNAWALI et al. 2011). However, the actual distribution of fishing cat is not well understood in Nepal. Most of the fishing cats presence information is based on opportunistic records during the surveys targeted at large flagship species like tigers *Panthera tigris* (YADAV et al. 2018, POUDEL et al. 2019, TIMILSINA et al. 2021).

The evidence of fishing cats have been recorded from five protected areas of Nepal namely Koshi Tappu Wildlife Reserve, Chitwan National Park, Bardia National Park, Shuklaphanta National Park and Parsa National Park and three other sites (namely Jagadishpur Reservoir of Kapilvastu, Sunsari and Bara districts) outside of the protected areas in Nepal's Terai region (MISHRA et al. 2021). Around 70% of fishing cat range lies outside the protected areas in Nepal (MISHRA et al. 2022). Fishing cat is a habitat specialist, prefers wetlands such as water bodies, swamps and marshes with dense tall grassland (MISHRA et al. 2018, MUKHERJEE et al. 2012) and areas with culverts and bridges nearby water bodies (MUKHERJEE et al. 2012). Fish are the major diet of fishing cat with contribution

over 70% (SUNQUIST and SUNQUIST 2002) followed by birds and insects (MYERS et al. 2006). Occasionally, they may prey on small civets, young fawns of spotted deer, wild pigs, domestic goats, calves, dogs, poultry and water fowls where sometime seen scavenging tiger kills and livestock carcasses (NOWELL and JACKSON 1996).

This wetland dependent species is vulnerable to habitat loss and degradation as wetlands are most threatened ecosystem globally (DAVIDSON et al. 2018). Poaching, retaliatory killing, guard dog, movement of cats and livestock around the fish farm as well as core areas and nowadays road accidents are also reported as the major threats to fishing cat throughout its distributional range (MUKHERJEE et al. 2016, MISHRA et al. 2021).

Globally, fishing cat population has declined by 30% by last 15 years and is projected to decline by 30% in upcoming time (MUKHERJEE et al. 2016). In Nepal, detailed information on the distribution and status of small cats is sparse. Little information is available based on the historic references and signs survey designated for large felids in protected areas of Nepal (POUDEL et al. 2019). Also, research priorities are slanted towards big cats despite of the feline richness in the country (ARYAL et al. 2018). In this paper, we assess the distribution status, habitat use and major conservation threats that are impending the survival of this charismatic species in Shuklaphanta National Park (ShNP) of Western Nepal. Our study may contribute to know about the current distribution and habitat use of fishing cat in ShNP. This study can also be the basis to open up the further research areas using evidence based photographic and genetic level studies and developing appropriate conservation plan for the long term conservation of this threatened species.

Materials and Methods

Study area

We conducted our study in the core area of Shuklaphanta National Park (ShNP), which is located at the southwestern corner of Sudurpaschim Province of Kanchanpur district in Nepal (latitude – 28°50′25″N and longitude – 80°13′44″E) – Figure 1. It covers an area of 305 km^2 extending from an altitude of 174 to 1,386 m from mean sea level. It was gazetted in 1976 as Royal Shuklaphanta Wildlife Reserve and later in 2017; it was converted into national park. The area of 243.5 km^2 surrounding the reserve was declared as buffer zone in 2004 and it is comprised of mainly agriculture land (70%) followed by forests (21%), water bodies (7%) and grasslands (2%) (ShNP 2017).

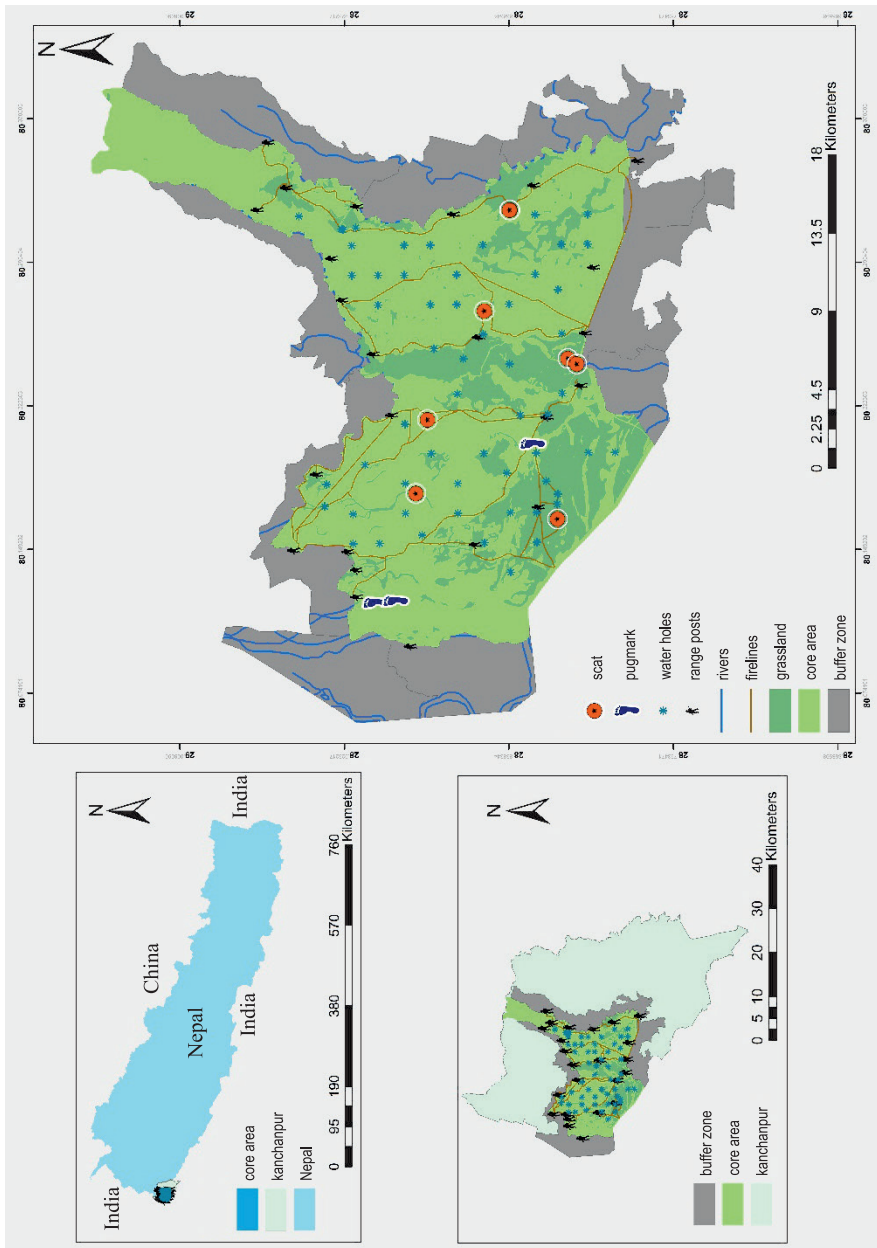

Fig. 1. The study area map showing park core area and buffer zone along with major land cover types and the location of fishing cat's scats and pugmarks detected during field survey 2019

It is bounded by the Syali River in the east, Mahakali in the west, Siwalik Hills in the north and east, and the Lagga Bagga forests and grasslands of Pilibhit Tiger Reserve lie in the Indian side in the south (DNPWC 2017). The park is an important part of the Terai-Duar Savanna and Grasslands eco-region with the major grass species are *Imperata cylindrica, Heteropogon contortus, Phragmites karka* and *Saccharum spontaneum* and this vegetation covers about 27% of the park's total area (ShNP 2017). The majority of park area is covered by the forests (60%) with Sal *(Shorea robusta)* dominant forest and other associated trees include *Terminalia alata, Largestromia parviflora* and *Pterocarpus marsupium* and other riverine forests such as Khair *(Acacia catechu)* and Sissoo *(Dalbergia sisoo)* (DNPWC 2017). The park provides prime habitat to globally threatened species such as Asiatic elephant *(Elephas maximus)*, one-horned rhinoceros *(Rhinoceros unicornis)*, Royal Bengal tiger *(Panthera tigris)*, sloth bear *(Ursus ursinus)*, smooth-coated otter *(Lutrogale perspicillata)*, fishing cat *(Prionailurus viverrinus)*, hispid hare (*Caprolagus hispidus),* hog deer (*Axis porcinus*) and rusty-spotted cat *(Prionailurus rubiginosus)* (DNPWC 2017, SADADEV et al. 2021).

Data collection

The field study was carried out between February and March 2019. Preliminary survey was carried out to identify potential habitats used by fishing cat in Shuklaphanta National Park before starting the actual field work. In consultation with the concerned stakeholders (park warden, experienced park staffs, local fishermen and nature guides), areas around Mahakali river, Baba Tal, Rani Tal, Salgaudi Tal, Kalikeech Tal, Sighpur, Pipariya, Shuklaphanta, Tara Tal, Chaudaha River, Kuwa Dada, Ghumauna, Bannikheda, Kapton Ghat inside national park were selected as potential sites for fishing cat survey.

Transect walk

For recording fishing cat distribution, we used a transect survey – a method widely used to monitor large mammals and felid species. Altogether 14 strip transects with length ranging between 1–2 km and 5 m width were laid randomly along the walking trails and existing paths of the park where direct sightings of fishing cat frequently detected by the park authority and the nature guides (Supplementary Information – Tab. Appx. 1). Transect walks followed major potential habitat types, viz waterholes, river or stream banks, marshy areas with grassland. Two co-authors and one experienced park staff walked slowly through each

transect to locate and identify the direct (direct sightings) and indirect signs (scats, pug marks) for consideration of fishing cat distribution in the study area. In each transect, transect number, site name, length and direction of transect, GPS location of starting and end point of transects, major vegetation types were recorded in the survey forms. Information on signs site, sign types (scats and pugmarks) were also recorded and also took their photographs for further validation. Spotted scats were identified on the basis of scraps, size, color, undigested prey remains in the feces, location and tracks followed by fishing cats based on local knowledge and photographs. Pugmarks were distinguished by visually observing the evidences of fish scales along the tracks followed by the fishing cat or nearby the spotted pugmarks.

Habitat use mapping

Habitat parameters (such as forest types, grasslands, shrub lands, cultivated lands, sand banks, river, streams, waterholes, etc) were recorded at the location of transects where signs (scats and pugmarks) or direct sighting of fishing cat were encountered. To quantify the habitat types used by fishing cat, we utilized the land cover of Nepal 2010 (ICIMOD 2013) and created a buffer of 1.5 km from the point of fresh scat and pugmarks collected from the field in accordance to the home range of fishing cat (SUNQUIST and SUNQUIST 2002). The habitat types within the buffer area were quantified using Arc GIS 10.8.

Threat assessment

Key informant interviews (n = 11) (7 park staffs, 3 National Trust for Nature Conservation staffs and 2 nature guides) were conducted to identify the major conservation threats to fishing cat which were further verified through the direct field visits and were ranked with relative threat ranking method (*Resources for implementing…* 2007) following (CHHETRI et al. 2020). To understand and quantify the major threats three criteria scope, severity and urgency were used (*Resources for implementing…* 2007).

Data analysis

Field data were analyzed using MS Excel and distribution map of the fishing cat was produced on the basis of available signs data (pugmarks and scats) using ArcGIS 10.8 version. Scats were identified by utilizing local knowledge and expert judgment (two co-authors and one experienced park staff and consultation of experienced nature guides) on the basis of

scraps, size, color, undigested prey remains in the feces, location and tracks followed by fishing cats based on knowledge and photographs. Similarly, pugmarks were identified on the basis of size and the evidences of fish scales along the tracks followed by the fishing cat or nearby its pugmarks. Major habitat types used by the fishing cat within the 1.5 km buffer were analyzed and presented as frequencies and percentages. Similarly, for conservation threat assessment, a relative threat ranking method was used (*Resources for implementing…* 2007, KAFLE et al. 2020) and three scales of classification – scope, severity and urgency were used to identify and rank the major existing threats. Three criteria were assigned to each of the identified issues and allotted a relative rank from high (5) to low (1) based on scales of WWF (2007), and finally, it was reclassified into 4 sub-classes very high, high, moderate and low (Tab. 1).

Table 1

Scales of ranking of scope, severity and urgency in relative threat ranking adapted from WWF (2007)

Criteria and rankings	Definition
Scope	the geographical scope of impact on the biological target that can reasonably be expected within 10 years under current circumstances
Very high	the threat is likely to be pervasive in its scope, affecting the target across all or most (71–100%) of its occurrence/population
High	the threat is likely to be widespread in its scope, affecting the target across much (31–70%) of its occurrence/population
Medium	the threat is likely to be restricted in its scope, affecting the target across some (11–30%) of its occurrence/population
Low	the threat is likely to be very narrow in its scope, affecting the target across a small part (1–10%) of its occurrence/population
Severity	the level of damage to the biological target that can reasonably be expected within 50 years under current circumstances
Very high	within the scope, the threat is likely to destroy or eliminate the target or reduce its population by 71–100% within 10 years or 3 generations
High	within the scope, the threat is likely to seriously degrade/reduce the target or reduce its population by 31–70% within 10 years or 3 generations
Medium	within the scope, the threat is likely to moderately degrade/reduce the target or reduce its population by 11–30% within 10 years or 3 generations
Low	within the scope, the threat is likely to only slightly degrade/reduce the target or reduce its population by 1–10% within 10 years or 3 generations
Urgency	this characteristic is used to assess the certainty and time scale over which impacts of the threat will be observable

cont. Table 1

Very high	the effects of the threat are already observable and there is an importance to take action to deal with the threat within a year
High	the effects of the threat are likely to occur and the threats are expected within the next 1–10 years
Medium	the effects of the threat are likely to occur and the threats are expected within the next 10–25 years
Low	the effects of the threat are unlikely to occur and the threats are expected in about 25 years from now

Results

Distribution of fishing cat

We found that there was not any direct sightings of fishing cat during the entire field work period. So we considered two kinds of indirect signs i.e., scats and pugmark as a presence of fishing cat in ShNP. All together 10 indirect signs were detected along the 8 transects out of 14 transects. Among them, fresh scats (n = 3) and pugmarks (n = 2) were detected from transect 6, 9 and 11 (supplementary information – Tab. Appx. 2), where the frequent sightings of fishing cat were also recorded by the park authority while doing daily patrolling operations and nature guides. The majority of signs (n = 7) were distributed in nearby the wetland with sparse sal forest, riverine forest and edge of grassland with marshy areas (Fig. 1, supplementary information – Tab. Appx. 2).

Habitat use of fishing cat

The majority of signs were detected on animal trails at the edge (within 30 m) of waterholes, marshy areas, riverine forest and grassland with sparse sal forest (Fig. 2, supplementary information – Tab. Appx. 2). The results conclude that major habitat characteristics used by fishing cat include grasslands (30.6%), cultivated land (21.77%) and forests (21.14%) respectively (Fig. 2, Tab. 2).

Table 2

Details of habitat within 1.5 km radius of fishing cat's fresh signs detected along the transects in Shuklaphanta National Park, Nepal

Transects	Site name	Habitat types area in hectares within 1.5 km radius of fresh scat and pugmarks detected sites						
		forest	shrub land	grass-land	swamp area (wetland)	culti-vated land	water bodies	sandy area (river bank)
Transect 6	Pipariya	169.80	120.91	–	1.23	109.21	135.40	170.23
Transect 9	Shuklaphanta	–	–	647.57	–	–	59.22	–
Transect 11	Tara taal	278.33	68.80	1.56	–	352.45	1.71	3.87
Total		448.13	189.71	649.13	1.23	461.66	196.33	174.10
		21.14%	8.95%	30.6%	0.06%	21.77%	9.26%	8.21%

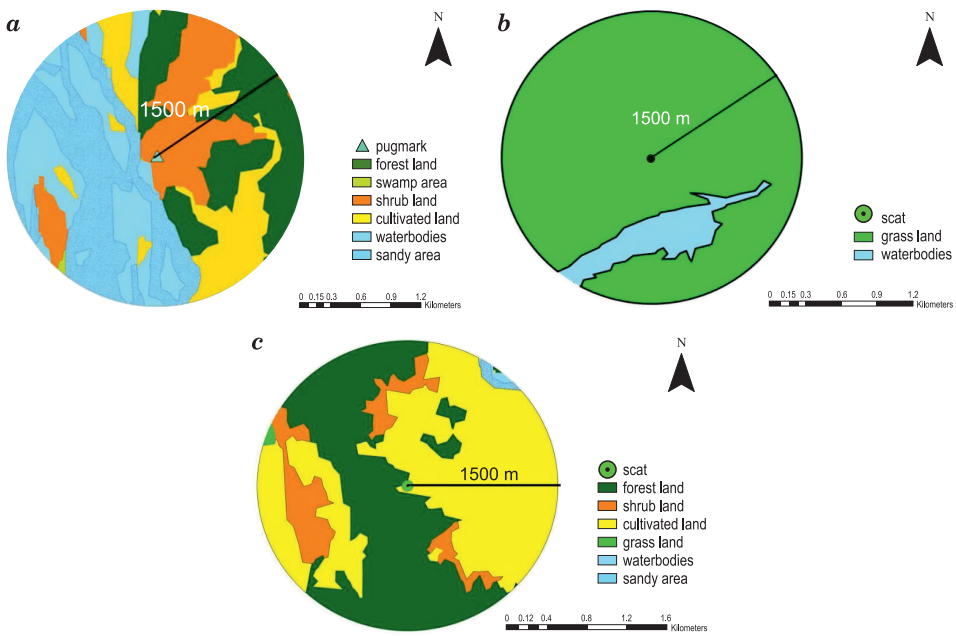

Fig. 2. Habitat use map of fishing cat within 1.5 km radius of signs detected sites along transect 6 (*a*), transect 9 (*b*) and transect 11 (*c*)

Conservation threats of fishing cat

Among five threats identified during the field survey and key informant interviews, over fishing (31.11%), wetland depletion (28.89%) and lack of recognition of fishing cat habitat (20%) are the most prominent issues as very high ranked threats of fishing cat in the study area (Tab. 3). A detailed ranking classes of all five main threats are shown in Table 3.

Table 3
Relative ranking of threats to fishing cat in Shuklaphanta National Park, Nepal

Threats	Scope	Severity	Urgency	Total	Percentage [%]	Threat category
Wetland depletion	5	4	4	13	28.89	very high
Over fishing	4	5	5	14	31.11	very high
Electrocution or poisoning	1	2	2	5	11.11	high
Human influence	2	1	1	4	8.89	moderate
Lack of recognition of fishing cat habitat	3	3	3	9	20.00	very high
Total	15	15	15	45	100.00	–

Discussion

Fishing cats were majorly distributed in and around wetland habitats in the patchy form. The indirect signs, i.e. scat and pugmarks were observed in those areas where the bushes and water bodies were abundant. Previous studies suggested that fishing cat prefer open structured features, water bodies with dense tall grassland (MISHRA et al. 2012) and areas with culverts and bridges nearby water bodies (MUKHERJEE et al. 2012), and our findings are consistent with those studies. The reason could be that the dense tall grassland and bushes help them to hide from their predators and might have used these habitats for shade and prey on grassland birds since, fishing cat are found to prey on birds and rodent species occasionally (HAQUE and VIJAYAN 1993). Further, about 70% of their diet is composed of fish (SUNQUIST and SUNQUIST 2002) and this could be the reason to live in the proximity of water bodies. Our study indicated that sal forest, riverine forest, sandy area or river banks, grassland with marshy area and wetland with sparse sal forests are the major habitat types used by the fishing cat. These results coincide with the results of (MUKHERJEE et al. 2016) where they found that sub-tropical forest areas, tall and short grasslands (flooded), wetlands, marshlands were found to be the major habitat. However, with the limitation we used sign survey method (scats and pugmarks) by utilizing the local field knowledge and expert judgment of Shuklaphanta National Park staffs and senior nature guides who are familiar to the area, which might not accurately represent their actual distribution and habitat use in ShNP as there are other sympatric small carnivores occurrence in the area. Further our study revealed that over fishing, wetland depletion, lack of recognition of fishing cat habi-

tat, electrocution or fish poisoning and human influence as existing severe threat in and around the national park which are similar to the study carried out by (MISHRA 2013, MISHRA et al. 2021). DAVIDSON et al. (2018) reported that wetland ecosystem is the most threatened ecosystem worldwide which makes fishing cat vulnerable. Likewise, electrocution or poisoning in the privately owned fish ponds or aquaculture by local communities in the eastern Nepal to are known to retaliate fishing cat to death (TAYLOR et al. 2016, MISHRA et al. 2021) however none of such death cases reported in our study. Shrinkage of wetlands, flooding and exploitation of riparian vegetation by the grazing livestock are identified as a threat by (TAYLOR et al. 2016, MISHRA et al. 2018) which supports our study. Human disturbance and over fishing were also major threats to the species as highlighted by SUNQUIST and SUNQUIST (2002), CUTTER and CUTTER (2009) which are consistent to our study.

Conclusion

Our study concludes that the fishing cat is distributed in and around the wetland habitat in the patchy form. Further, our study identified sal forest, riverine forest, sandy area or river banks, grasslands with marshy area and wetland with sparse sal forest as major habitat for the fishing cat. The major threat that are impending the survival of fishing cat in and around the study area were identified as over fishing, wetland depletion, lack of recognition of fishing cat habitat, electrocution or poisoning and human influence. Conservation of wetland habitats, increase fish stock in the wetlands and conservation awareness programs focusing fishing cat distribution sites should be included in ShNP conservation planning to ensure their survival. We also recommend the evidence based photographic capture-recapture survey and genetic level study to determine the actual status and distribution of fishing cat as well as habitat use by GPS tagging in ShNP.

Accepted for print 11.07.2022

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Appendix

Table Appx. 1

Detailed information of 14 transects laid down in Shuklaphanta National Park, Nepal

Transect name	Site name	Transect length [m]
Transect 1	Baba Tal	1120
Transect 2	Baba Tal	1020
Transect 3	Salgaudi Tal	1070
Transect 4	Rani Tal	1540
Transect 5	Sighpur	1300
Transect 6	Pipariya	2000
Transect 7	Kapton Ghat	1250
Transect 8	Silalake chowk, pillar No 24	1290
Transect 9	Shuklaphanta	1000
Transect 10	Baba Tal to Kuwadada	1400
Transect 11	Tara Tal	1020
Transect 12	Between Chaudaha river and kalikeech Tal	1640
Transect 13	Ghumauna Tal	1310
Transect 14	Bannikheda (Chaudaha River side)	1070

Table Appx. 2
Details of indirect signs detected location during transect walk (February-March, 2019) along with site descriptions in Shuklaphanta National Park, Nepal

Transect	Sign number	Site name	Habitat type	Dominant vegetation	Ground cover	Distance from water source [m]
Transect 3	1 Scat	Salgaudi	Riverine Forest	*Syzygium cumini*	Fiddlehead Fern, Calamus	100
Transect 5	1 Pugmark	Sighpur	Sal Forest	*Shorea robusta*	*Imperata cylindrica,* Naranga, *Citrus maxima*	30
Transect 6	2 Pugmark	Pipariya	Riverine Forest	*Syzygium cumini*	*Imperata cylindrica, Citrus maxima, Ageratina adenophora*	0.5
Transect 9	1 Scat	Shuklaphanta	Grassland (Marshy area)	*Citrus maxima*	*Imperata cylindrica, Citrus maxima*	150
Transect 10	2 Scat	Baba Tal	Sal Forest, Grassland	*Shorea robusta, Calotropis gigantea*	*Imperata cylindrica*, Ekri, Cimpokokan	23
Transect 11	2 Scat	Tara Tal	Wetland, Sal Forest	*Shorea robusta, Centella asiatica*	*Centella asiatica*, *Cynodon dactylon*, *Ageratina adenophora*	5
Transect 12	1 Scat	Chaudaha river	River bank	*Ipomoea carnea*	*Ipomoea carnea*	50
Transect 13	1 Scat	Ghumauna Tal	Riverine forest	–	Fiddlehead Fern, Calamus	10

POLISH JOURNAL OF NATURAL SCIENCES
Abbrev.: Pol. J. Natur. Sc., Vol 37(2): 233–262, Y. 2022

DOI: 10.31648/pjns.7414

A GRAPHICAL APPROACH FOR ANALYSES OF DATA THIN NON-PARAMETRIC CONTINUOUS VARIABLE OF *BOTIA DARIO* WITH R PROGRAMMING LANGUAGE

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Key words: graphicacy, dot chart, gaussian kernel, sheather-jones bandwidth, distribution pattern.

Abstract

Botia dario is categorized as endangered due to considerable drop in population over past two decades creates data thin condition where the nonparametric statistical methods are superior alternative approach for data drafting. Small data set necessitate graphical display reducing the chance of data compression by numerical analyses. Monthly mass and length density estimates, location, and spread were compared with R computing environment through charts and plots; proposing a graphical method for single discrete and continuous data analyses. Through graphicacy the novel method reveals the pattern for mass and length of *B. dario* by depicting the modes and skews of the kernel density estimates suggested wide fluctuations during pre-monsoon months; whereas the spreads and locations of boxplots draping dot-whiskers infer the Gaussian kernel by pairwise comparisons. The boxplot widths, notches of the boxplots and red dot-whiskers illustrate comprehensive variations. The novel method and suggestive narratives appeal for inclusive use excluding the limitations.

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Introduction

Literacy (communicate with written information), numeracy (communicate with numerical information) and graphicacy (communicate with graphical information) are the central ability of human cognitive inheritance (KEEN 2010). Perhaps the latter came first, as the ancient cave paintings indicated. Statistical data should be presented in such that it consumes little space and ink (TUFTE 2001), and at the same time accumulate "more relevant information within eye span" "to make a contrast, a comparison, a choice" (TUFTE 1990). Data reduction (calculating the central tendency and dispersion) based on numerical information is a common statistical process which can be balanced through graphical methods avoiding probable information loss and maintaining data expression (KEEN 2010). Data compression methods like mean could sometimes lead to misinterpretation of the population due to reduced number of sample and/or sampling error; commonly are being faced for some fish species whose numbers are dwindling and very difficult to capture, resulting poor representation of the population. On the other hand, graph can communicate most efficiently and represents the sample data as a whole to make an effective conclusion. The insights of graphicacy allow the observers to adhere coherent information from a chart and/or a plot precisely than a numerical table.

The Queen loach *Botia dario* (HAMILTON 1822) is a very popular SIS (Small Indigenous Species) fetching higher price in Bangladesh. Unfortunately, the natural population of the species is under threat as the population noticeably has dropped over the past two decades and now being considered as an 'endangered' species (IUCN Bangladesh 2015). Not only *B. dario* but a total of 64 fish species are being facing different levels of threats in Bangladesh (NEUMANN and ALLEN 2007) which ultimately demands more research on those to restore the population and at the same time, forces the researcher to work with small number of individuals (sample). Sometimes, it was quite difficult to collect representative samples of specific categorical variable while considering natural populations. A parametric statistical method based on poorly representable sample (without bootstrap or Monte Carlo simulations) might mislead succeeding models and inferences. An exploratory plot for the distribution of single continuous variable is empirical distribution function (EDF). The length frequency distributions are popularly presented in the fisheries literatures with the Kolmogorov-Smirnov (K-S) two-sample test (NEUMANN and ALLEN 2007) which determines whether the EDF are similar between groups or significantly different in the location (central tendency), spread

(data dispersion) and shape of the distributions (HOLLANDER et al. 2014). Again, if the discrete variable had more groups, then the pairwise comparison is strenuous to explain. For example, if the complied data require twelve groups' pairwise comparison (as in this study), then there are sixty-six *p*-values obtained by K-S two-sample test. Furthermore, when the comparison is extended to compare with the global location; at that point additional twelve pairwise comparisons would have added with mounting difficulties to conclude fairly. This numerical convention for data analysis might be replaced by graphical convenience smoothly. Therefore, Kernel density estimate – a nonparametric method (without assuming a model and estimating parameters from data), is probably the best alternative for single continuous variable; while variable width notched boxplots and dot whisker plots could be the best choice for comparing among categorical group variables. BURN (1993) offers an immaculate mnemonic, ACCENT for six principles (apprehension; clarity; consistency; efficiency; necessity; and truthfulness) of effective graphical communication. For producing effective graph following the ACCENT rules R programming languages (R Core Team 2021) with versatile packages can act as a prospective tool. Moreover, the inherent properties of R help to inspect the codes of complex statistical concepts step by step to get the results and the graphical output as well (AHO 2014 on the Preface page xix).

Although, there are few studies have been conducted on conservational view of *B. dario* (RAHMAN 2015, HOSSAIN et al. 2015, MAHFUJ et al. 2022), stock structure (MAHFUJ et al. 2022), and on sex ratio and fecundity (HUSSAIN 2007) but authors failed to found any on nonparametric method for any parameter of this species; ultimately attracted the authors to use the species as a model in this work. Conceiving a novel nonparametric approach with R for single continuous variables (mass and length of *B. dario*) separately compared by one discrete grouping variable (months) is imperious to transform conventional numerical analysis into effective graphical platform. The novel approach allows readers, researchers, and planners to get coherent information from small data set through the primitive inherent graphicacy and to conclude from heuristic to holistic one.

Materials and Methods

Study site, sample collection and recording of mass and length

Samples were collected 3 points of the 'Sunamganj Beel' (a lake-like wetlands with static waters might have channel to connect to river) namely 'Dakshin Sunamganj' (24°55'52.0"N 91°26'53.2"E), 'Balaganj' (24°39'13.1"N 91°49'35.8"E) and 'Raznogor' (24°39'08.9"N 91°49'55.1"E) of northeastern Bangladesh during August 2018 to July 2019. HAMILTON (1822) recorded the first specimen of *B. dario* from the northern rivers of Bengal and latter all other taxonomists (TALWAR and JHINGRAN 1991, RAHMAN 2005, RAHMAN and RUMA 2007) described this region as native of *B. dario*. The three points are connected by the Kushiyara River with its tributaries and distributaries. Moreover, the 'Sunamganj Beel' is usually inundated in the monsoon, seemingly the entire area under water. Therefore, the site was considered to collect the species. A total of 226 individuals were captured by fine meshed seine net (Bengali name: Kapuri jal) and were preserved immediately with 10% formalin solution to avoid any spoilage. The samples were then transported to the laboratory of the Department of Fisheries and Marine Science, Noakhali Science and Technology University for further analysis. Mass and length were measured as whole-body mass to the nearest 0.01 g by an electrical balance (EK600 Dual) and total length (TL) to nearest 0.01 cm using a digital slide calipers having ±0.01 mm accuracy (EAGems-B00Z5KETD4) following the instructions of JENNINGS et al. (2012).

Months (discrete grouping variable)

Although the sampling was started in August 2018 and closed in July 2019, the order of months in the graphical displays (charts and plots) were presented as March to February following the rain dependent seasonal pattern viz., Mar.–May (pre-monsoon), June–Aug. (monsoon), Sept.–Nov. (post-monsoon) and Dec.–Feb. (winter).

Dot chart

The dot chart (Fig. 1) was constructed sharing (with required modifications) the codes of KEEN 2010 which followed the inferred plotting standard of CLEVELAND and McGILL 1984. The modifications were:

1. Ticks on the vertical axis for clear linking with data point to corresponding group labels.

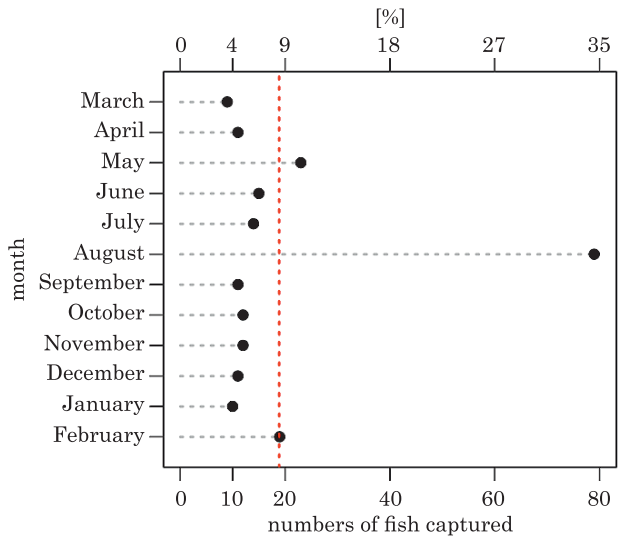

Fig. 1. Dot chart for numbers and percentages of *B. dario* captured from 'Sunamganj Beel' from August 2018 to July 2019. Month's order (top to bottom) started at the onset of pre-monsoon and end at the end of winter season. Vertical dashed red line indicating the mean of total captured. Ticks added on the horizontal axis at the data dense zone

2. Additional horizontal axis on top coordinate to see corresponding percentage of the values on the bottom coordinate.

3. Vertical dotted red line to mark the mean level of the count.

4. Additional ticks on both horizontal axes to compare the dot dense region.

5. Y-axis label to indicate the discrete grouping variable. The detailed code script is attached in Appendix 1.

Trellis kernel density plots

Kernel density plots (Fig. 2 and Fig. 3) were produced using `densityplot()` function of **lattice** package with the syntaxes `kernel="gaussian"` for Gaussian kernel estimation and `plot.points="rug"` for adding rug on horizontal axis beneath the curve. WAND and JONES (1995) suggested objective method (automatic data dependent) for selecting bandwidth (`bw.SJ()` SHEATHER and JONES (1991) were applied as it was explained that the subjective selection was time consuming and there was no idea about the outline of the distribution. The detailed code script is obtainable in Appendix 2 and Appendix 3.

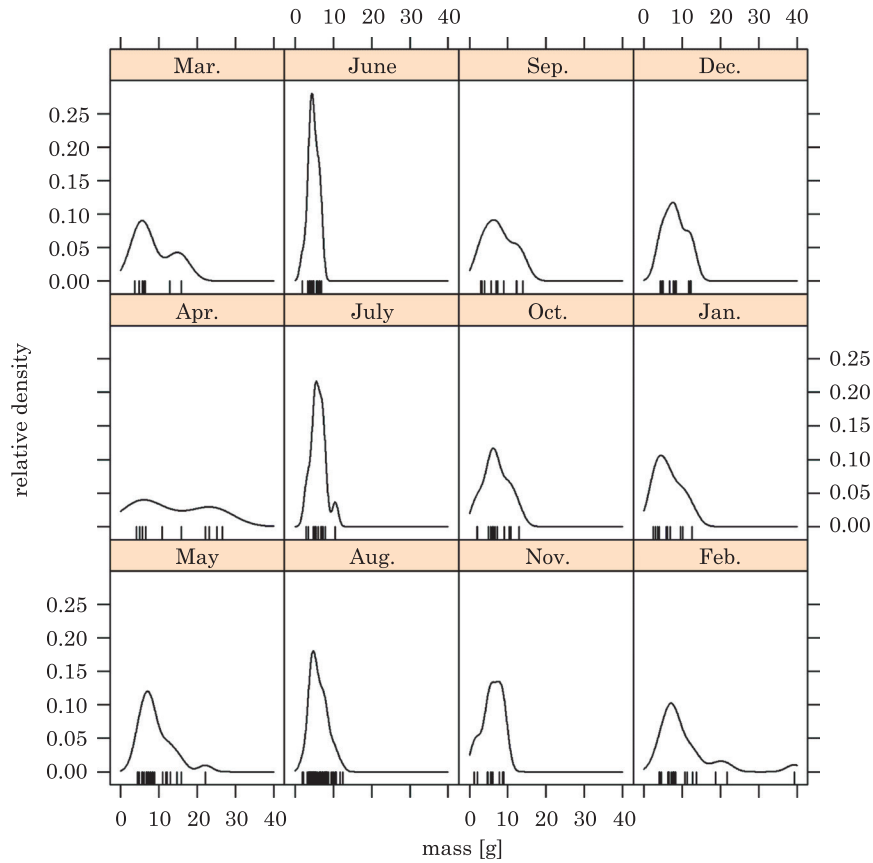

Fig. 2. Nonparametric kernel density plots for mass [g] of *B. dario* captured from 'Sunamganj Beel' from August 2018 to July 2019. The short names of the months in the strips ordered column--wise (top to bottom then to right). Each column represents a season viz. pre-monsoon, monsoon, post-monsoon and winter. Gaussian kernel with data dependent bandwidth is used to estimate the relative density

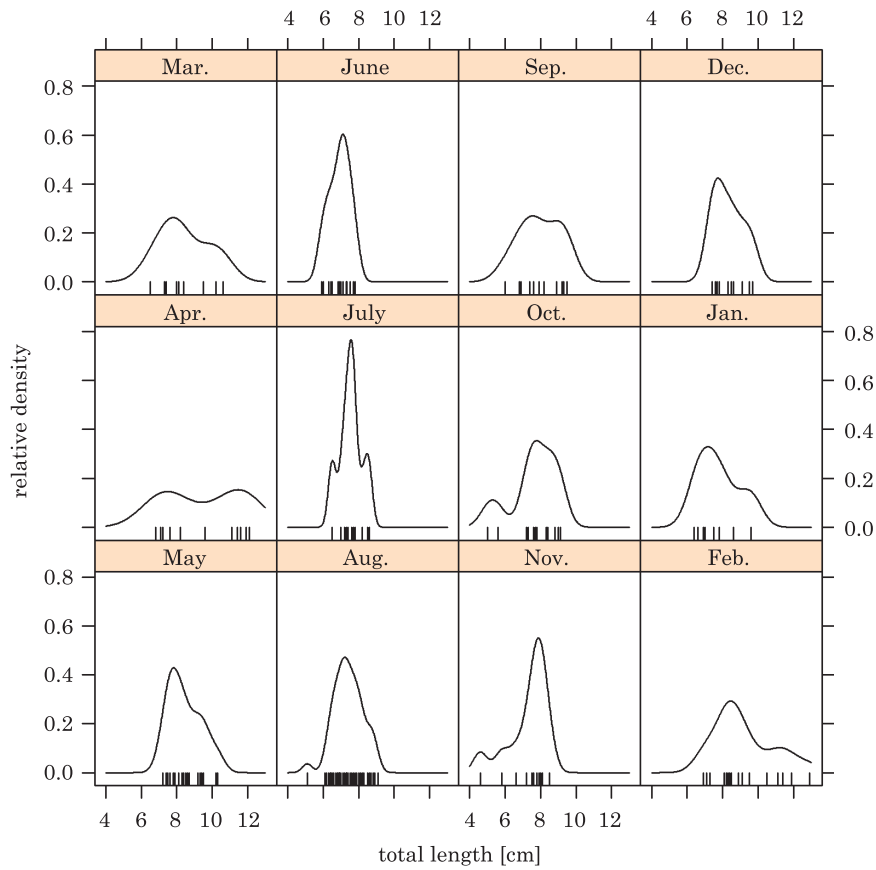

Fig. 3. Nonparametric kernel density plots for length [cm] of *B. dario* captured from 'Sunamganj Beel' from August 2018 to July 2019. The short names of the months in the strips ordered column-wise (top to bottom then to right). Each column represents a season viz. pre-monsoon, monsoon, post-monsoon and winter. Gaussian kernel with data dependent bandwidth is used to estimate the relative density

Variable-width notched boxplot draping dot-whiskers

Variable-width notched outlier boxplots draping dot-whiskers (Fig. 4 and Fig. 5) were constructed using the codes of KEEN (2010) (function `stripchart()` was omitted, as `boxplot()` call active) after some modifications. The modifications were:

1. Variable-width notched boxplot and dot-whisker plot drafted within a single frame to compare between the locations (median and mean) and their spreads (standard error) simultaneously; and to assume their skew (without observing the density plot).

2. Total sample plotted (box plot, `add=TRUE` syntax added in the second plot call) with a light gray separation line as same visual like the box plot to compare between each group and total.

3. For both locations a reference line (median and mean of total marked by vertical dotted black line and red line, respectively) drawn to compare among each groups fairly definite with an ease.

4. Ticks added on the horizontal axis at a rough start (Fig. 5) and end (Fig. 4 and Fig. 5) of the values of observations to predict the ranges.

5. Logarithmic scale (argument `log="x"` inside plot call) added for the mass on the Figure 4 for superior comparison. The detailed code script is accessible in Appendix 4 and Appendix 5.

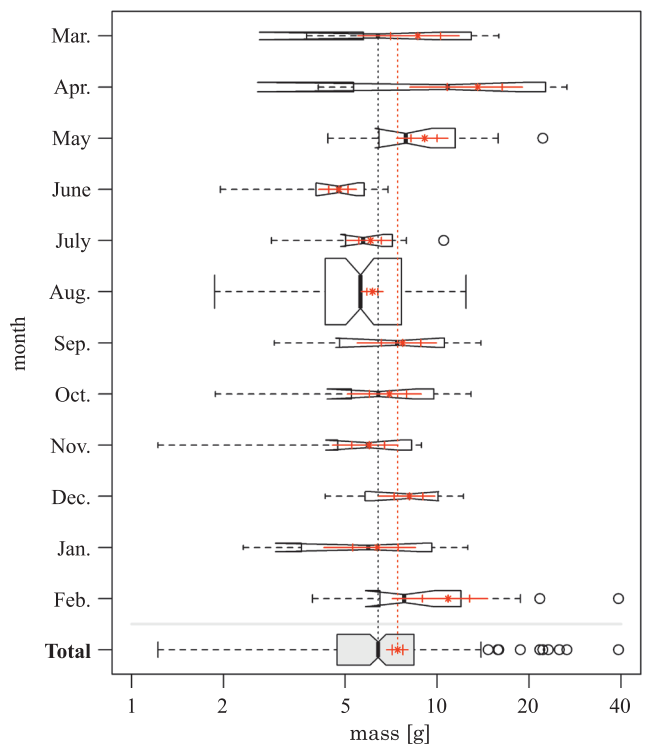

Fig. 4. Variable-width notched boxplot (box width proportional to sample size, except for the total, which was added with the syntax `add = TRUE`, where the proportion argument is inapplicable) draping dot-whiskers for monthly mass [g] with logarithmic scale of *B. dario* caught in 'Sunamganj Beel' from August 2018 to July 2019

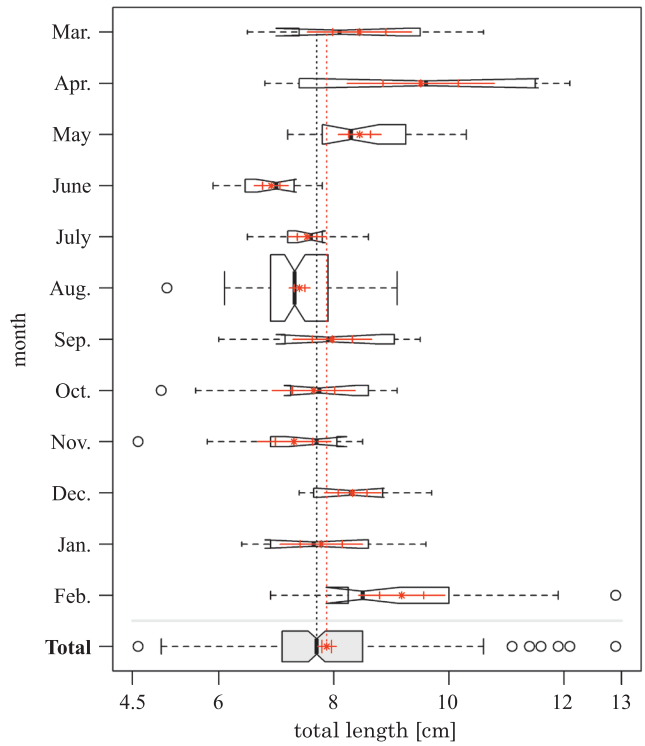

Fig. 5. Variable-width notched boxplot (box width proportional to sample size, except for the total, which is added with the syntax `add = TRUE`, where the proportion argument is inapplicable) draping dot-whiskers for monthly length [cm] of *B. dario* caught in 'Sunamganj Beel' from August 2018 to July 2019

Data analyses

Raw data were entered into a spreadsheet (MS Excel 2016) and save as .csv file extension. The CSV file act as data frame (accessible in Appendix 5 – Table Appx. 5.1) for further data analyses using the R programming language (R Core Team 2021). Executions of basic stats were performed using core R, **dplyr** (WICKHAM et al. 2021) and **FSA** (OGLE et al. 2021). Package **graphics** (built in base R) and **lattice** (SARKAR 2008) were used to create and to modify charts and plots.

Results

Dot chart

Figure 1 is the dot chart corresponding the number of *B. dario* caught from 'Sunamganj Beel' by month. The highest occurrence (capture) was recorded on August (nearly 35%) and the lowest on March (nearly 4%). Only three points i.e., May, August and February were above to right) the mean of total captured.

Trellis kernel density plots

Nonparametric kernel density plots for both mass (Fig. 2) and length (Fig. 3) exhibited pattern similarities with minor modal variations in column two to four. Those columns represented monsoon, post-monsoon and winter season, respectively. Most of the captured individuals during monsoon were found around 5 g (mass) and below 8 cm (length) constructing high narrow peaks for the both kernel density estimates indicating spawning came about at the end of pre-monsoon.

On winter season, although the majority of observed mass showed similarities with monsoon and post-monsoon but the length kernel density estimate made a bolder right skewed mode of above 8 cm. Considering the mass kernel density estimate, there was another spawning season possible at late post-monsoon whereas, the length kernel density estimate suggested the stock is struggling for food and space on winter months. In the first column (pre-monsoon), the positive skewed mode of over 20 g (Fig. 2) and 10 cm (Fig. 3) individuals on March indicated the stock was preparing for spawning and on April, was fully prepared. Therefore, the second mode of those bimodal kernel density estimates indicated the gravid dames. The relatively wider left peak modes for both kernel density estimates on May signposted spawning has been started.

Variable-width notched boxplot draping dot-whiskers

Noticing both the plots (Fig. 4 and Fig. 5), it was observed that there were wide variations considering the standard deviation of the median (length of the notches) and standard error of the mean (red dot-whiskers) over the twelve months. Projecting the comparison of the notches for months it was found to be similar considering the median mass for all months except June. Whereas, all the months including the total were similar except June, August and February with respect to the median length ($p < 0.05$). Considering median mass, approaching from left to right

a similar group of nine months excluding May, December and February were observed, while moving from right to left a group of ten months except June and August was revealed. Repeating the same considering median length, starting from left to right a similar group of eight months excluding April (additional see mass), May, December and February was found. While moving from right to the left a group of nine months except June, July (addition, see mass) and August was revealed ($p < 0.05$).

On the other hand, all months including the total except April, June and August were found to be similar while projecting the comparison of the whiskers for months considering the mean mass. Whereas, all the months including the total were similar except April, May, June, August and February with respect to the mean length ($p < 0.05$). For mean mass, approaching from left to right a similar group of five months (June, July, October, November and January) and moving from right to the left a group of eight months (except June, July, August and November) were found. Repeating the same for mean length, a group of four months (June, October, November and January) and a group of seven months (except June, July, August, October and November) were revealed ($p < 0.05$).

Pairwise differences of locations (median by notches of the variable width boxplot and mean by red dot-whiskers) between months showed May and June were significantly ($p < 0.05$) different for both mass and length.

Length of the notches is centered about the sample median and overlapping notches for any pair of months indicated no differences in sample medians ($p < 0.05$). Dot-whiskers with red star dots give the locations for the sample means and the red bars sliced the red whiskers on both sides marked the length of one standard error, and overlapping red whiskers for any pair of months indicated no differences in sample means ($p < 0.05$).

Discussion

Although few other packages (ggplot2 (WICKHAM 2016), plotly (SIEVERT 2020), ggpubr (KASSAMBARA 2020) are available brilliantly to cope with R for plotting convention but in many cases the routine functions like `par ()`, `axis ()`, `points()` and `lines()` are not compatible with the high level plot functions of other packages. Those plot functions required their package specific syntaxes. That is why; the authors limit themselves within the base or core R and incorporation of the same function of other packages is beyond the scope of this article.

Dot chart

The peak count on August suggested the spawning of *B. dario* commenced prior to August, presumably at the end of pre-monsoon which was fairly impossible to conclude without the mass and length density estimates. There was a third peak on late winter and whether was it indicated another spawning incidence or higher fishing pressure as a result of lower water level or was there any monthly (temporal) pattern evident? We try to resolve the queries as discussion progress.

Considering the data points on the chart the reference vertical red dotted mean line acted as the balance of the total vales of the dashed gray line portion. The large percentages (nearly 35%) for August forced the majority of the points left to the mean line, which might be a poor representation of the data set. However, this sampling majority did not show any recognizable influence further except slim width of the other boxplots; while the nonparametric protocol for graphical convenience was applied. Most of the dots in the dot chart showed close vicinity of the mean while excluding the count of August. Moreover, KEEN 2010 stated the principle of the central limit theorem which suggested "even for long-tailed distributions with sample sizes as small as ten" sufficient for "quite good" approximation. In the current study the sample sizes were "as small as ten" in few months which might not apprehend "quite good" deduction as the central limit theorem comprehend.

The modified dot chart is proposed for easy comparisons between the count and the percentages of the count specifically indicating the ratio of total by two horizontal axes; and additional ticks on the data dense portions in horizontal coordinates for comparing among the groups of the discrete variable on the vertical coordinate. Dot plot tick for vertical categories were absent while CLEVELAND and MCGILL 1984 designed it whereas, the proposed dot chart recommended it for better linking with the gray dashed line terminating the dots. Moreover, the dot plot is in the top most position leaving bar chart, pictograph, pie chart etc. as KEEN 2010 suggested hierarchy of preference for single discrete variable.

Trellis kernel density plots

Nonparametric density estimates for monthly kernels pictured the seasonal influences over both mass and length giving a reasonably acceptable temporal pattern. Unimodal kernels of monsoon appeared to be smooth bimodal during post-monsoon and winter (except February) predicting a seasonal pattern similarity. Comparatively higher water levels during monsoon allowing the drifting stock more susceptible to be caught;

while progressing towards winter coupled with low water levels made possible to catch the bigger fish, as well. From February to May sharp decline of water level in Bangladesh context left the population into extreme fishing pressure depicted by the graduating right skewed bimodal curves.

The kernel density estimates were plotted four columns by three rows following the argument `layout=c(4,3)` inside the `densityplot()` function. The month order was rearranged for showing the seasonal progress from top to bottom to right. Conventionally, the lattice package arranges the strip categories from left to right to up. The future researchers need to arrange their own layout and order according to the data set and the way they ought to present.

For selecting the kernel density estimator (the kernel and the bandwidth) the current study shared the view and precautions discussed by KEEN (2010) and after a critical examination the estimator was chosen. For comparison, the authors suggest the scholars to replace the argument `kernel="gaussian"` by their desired one (`"epanechnikov"`, `"rectangular"`, `"triangular"`, `"biweight"`, `"cosine"` and `"optcosine"`). And, for the bandwidth, one can try to check the changes in the plot by inserting the `bw=value` argument inside the `densityplot()` call after obtaining the `value` (number) by the functions `bw.SJ`, `bw.nrd0`, `bw.nrd`, `bw.bcv` or `bw.ucv`. Running different combinations of estimators, the current study relied on the "## sensible automatic choice" (R Core team 2021). Researcher should be strictly cautious while changing the default to avoid drafting a plot like hornbill beak.

Variable-width notched boxplot draping dot-whiskers

Apart from finding the monotonous grouping similarities and pairwise comparisons in terms of spread with respect to the locations those plots (Fig. 4 and Fig. 5) might allow the spectator to pursue the overall scenario of the sampling distribution at a glance. The design of the plots discovered the layering and separation resemblances with the calligraphy of Uboku Nishitani when TUFTE (1990) wrote, "The saturated quality of the red partially offsets its lighter value and finer line (appropriate to meticulous annotation). Alone, each color makes a strong statement; together, a stronger one". In the graphical display the reader has the freedom of choice to compare locations, equate group ratio, form groups, match groups, identify skew, detect outliers, separate groups and might be more, "makes a strong *plot*; together, a stronger one".

In the boxplot draping the red dot-whiskers of November revealed an interesting puzzle. Putting the outlier inactive in the mass made a sym-

metric boxplot (in the logarithmic scale the left whisker appeared longer) by merging the locations seemingly within a point while, in the length the outlier act strongly by pulling mean apart from median. This marks the assumption about the second possible breeding during late post-monsoon under reconsideration.

The locations (median and mean) of mass was found the largest in April (although the outliers in February projected the larger individuals), while the smallest was in June. The scenarios were same for the locations of lengths except in November where the outlier represented the smallest individual.

Conclusion

By the course of time through the results and the discussions the novel graphical displays showed their statistical analytical capabilities to figure out the arising questions inferentially. For plotting single discrete variable, the dot chart with proposed modified design could be a superior alternative, which is sufficient to compare among group aggregated values or counts or proportions. With respect to current study, the counts with percentages allow one to decipher the monthly capture pattern comparing with the mean value. The trellis density plot requests the managers for improving the threat status of *B. dario* to take initiatives intensively during late dry to early wet by minimizing recruitment overfishing. The scholars have liberty to replace the kernel and bandwidths and have choice to rearrange the layout to make their own. The ability of the boxplot draping dot-whiskers shown by its comparison with level of significance and separation by location reference lines. The versatility of the plot allow the users to control the significance level, reshape the box, change the scale etc. by adjusting the spread (standard error) algorithms, by muting and allowing specific syntaxes to run. Hope the modified designs and the analytical narratives of the proposed charts and plots will be accepted for graphical displays of single discrete and continuous variables.

Applications and Limitations

The scripts (detailed codes) provided here with the supplementary .txt extension files are self-explanatory. The purposes of each succeeding codes were annotated with conventional #-marks. The readers have liberty to edit the code according to their requirements. The `par()` settings were

adjusted to accommodate the labels and leavings spaces for aesthetics; while, the `png()` function for the output image ratio. If the future user wish to control the plot ration rather than the image output then s/he would require to adjust the `pin`, `fin`, `mai` and `omi` arguments inside the `par()` function (MURRELL 2019).

Like other nonparametric methods this is robust for parametric applications also. Prediction of interactions among groups of a discrete variable is not possible through the novel graphical display.

Acknowledgements

The authors acknowledge the NATP-2, Bangladesh Agriculture Research Council, Dhaka, Bangladesh for the Research grants (NATP-2/PIU-BARC/Research CRG/2017/553) awarded during the study period. Authors would like to appreciate the efforts of the reviewers and offering their heartfelt gratitude to make the article better.

Accepted for print 27.05.2022

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Appendix 1

Supplementary file – Fig_1_dotchart.txt

```
# call file rest in working directory (wd)
dr <- read.csv("dario.csv")

# Ordered and factor
dr$Month <-
ordered(dr$Month,levels=c("Feb","Jan","Dec","Nov","Oct","Sep",
                          "Aug","Jul","Jun","May","Apr","Mar"))

# package required for new data frame
library(dplyr)
library(FSA)

# new data frame
tem <- dr %>% group_by(Month) %>%
  summarize(n=n(),val.n=validn(tl),
            mean=round(mean(tl,na.rm=TRUE),1)) %>%
  as.data.frame()

# For the dot plot new
NUM <- tem$n # x axis value
(mean1 <- mean(NUM)) # call the value of reference line

# for yaxis label

y <- c("February","January","December","November","October","Sep-
tember",
       "August","July","June","May","April","March")


# openning the printing device to make image as .jpeg
png("Fig_1_dotchart.jpeg",width = 5, height = 4.5,units = "in-
",res=588)

# change font
par(family="serif")
# par set
par(mar=c(bottom=3.9,left=6.9,top=3.9,right=1.3)+0.1)

# THE dot Chart of Cleveland and McGill
plot(NUM,y,type="n",xaxt="n",yaxt="n",xlim=c(0,80),
     ylim=c(0,length(y)+1), xlab="numbers of fish captured",
     ylab='',xaxs="r",yaxs="i")
for (i in 1:12) lines(x=c(0,NUM[i]),y=c(i,i),lty=3,l-
wd=1.8,col="gray")
```

```
points(x=NUM,y=1:12,pch=19,cex=1.0)
axis(1,at=20*(0:4),labels=TRUE,tick=TRUE,outer=FALSE)
axis(2,at=1:12,labels=y,tick=T,outer=FALSE,las=2,
     hadj=1,padj=0.3)

# adding extra horizontal axis
axis(3,at=20*(0:4),labels = round((20*(0:4))*100/226))
# addind axis level
mtext("[%]",side = 3,line = 3)
mtext("month",side = 2,line = 6)

# create vertical mean ref line
lines(c(mean1,mean1),c(0,length(y)+1),lty=3,lwd=1.8,col="red")

# additional ticks on side 1 and 3
axis(1,10)
axis(3,at=10,labels = round(10*100/226))

# image in wd
dev.off()
```

Appendix 2

Supplementary file – Fig_2_densityplotM.txt

```
# changing default font settings
font.settings <- list(
  font = 1,
cex = 1,
fontfamily = "serif")

my.theme <- list(
par.xlab.text = font.settings,
par.ylab.text = font.settings,
axis.text = font.settings,
sub.text = font.settings,
add.text = font.settings)

# call file rest in working directory (wd)
dr <- read.csv("dario.csv")

# ordering the strip names for layout=c(4,3)
dr$Month <- ordered(dr$Month,levels=c(
  "May","Aug","Nov","Feb",
  "Apr","Jul","Oct","Jan",
  "Mar","Jun","Sep","Dec"))

# package required
library("lattice")

# openning the printing device to make image as .jpeg
png("Fig_2_densityplotM.jpeg",width = 6, height = 6,units = "in",res=288)
```

```
# Mass plot call
densityplot( ~wt | Month,data=dr,xlab="mass [g]",
             type="density",kernel="gaussian",
             na.rm=TRUE,plot.points="rug",col="black",
             ylab="relative density",layout=c(4,3),from = 0,to=40,
             par.settings=my.theme)

# image in wd
dev.off()
```

Appendix 3

Supplementary file – Fig_3_densityplotL.txt

```
# changing default font settings
font.settings <- list(
font = 1,
cex = 1,
fontfamily = "serif")

my.theme <- list(
par.xlab.text = font.settings,
par.ylab.text = font.settings,
axis.text = font.settings,
sub.text = font.settings,
add.text = font.settings)

# call file rest in working directory (wd)
dr <- read.csv("dario.csv")

# ordering the strip names for layout=c(4,3)
dr$Month <- ordered(dr$Month,levels=c(
  "May","Aug","Nov","Feb",
  "Apr","Jul","Oct","Jan",
  "Mar","Jun","Sep","Dec"))

# package required
library("lattice")

# openning the printing device to make image as .jpeg
png("Fig_3_densityplotL.jpeg",width = 6, height = 6,units = "in",res=288)

# Length plot call
densityplot( ~tl | Month,data=dr,xlab="total length [cm]",
             type="density",kernel="gaussian",
             na.rm=TRUE,plot.points="rug",col="black",
             ylab="relative density",layout=c(4,3),from = 4,to=13,
             par.settings=my.theme)

# image in wd
dev.off()
```

Appendix 4

Supplementary file – Fig_4_boxplotM.txt

```
# call file rest in working directory (wd)
dr <- read.csv("dario.csv")

# ordering the grouping sequences
dr$Month <- ordered(dr$Month,levels=c("Feb","Jan","Dec",
                                      "Nov","Oct","Sep",
                                      "Aug","Jul","Jun",
                                      "May","Apr","Mar"))

# openning the printing device to make image as .jpeg
png("Fig_4_boxplotM.jpeg",width = 5, height = 6,units =
"in",res=588)

# changing default par settings
par(mar=c(bottom=3.9,left=3.9,top=1.3,right=1.3)+0.1)

# changing default font
par(family="serif")

# calculating the width ratio for boxplot
dr$Count<-dr$wt
dr$Count[dr$Count >= 0]<-1
dr$Count[is.na(dr$Count)== TRUE]<-0
sums<-tapply(dr[,"Count"],INDEX=dr[,"Month"],FUN=sum,na.rm=TRUE)
sums<-sums/max(sums)

# VARIABLE-WIDTH notched BOXPLOT call
boxplot(wt ~ Month,data=dr,ylim=c(1,40),xlim=c(0,12),
        xlab="mass [g]", ylab="month",
        names=levels(dr$Month),horizontal=TRUE,las=1,
        width=sums,notch=T,boxwex=1.3,col="white",log="x")

# calculating the mean and se for dot whiskers
means<-tapply(dr[,"wt"],INDEX=dr[,"Month"],FUN=mean,
              na.rm=TRUE)
sdf<-tapply(dr[,"wt"],INDEX=dr[,"Month"],FUN=sd,na.rm=TRUE)
dr$Iswna<-!is.na(dr$wt)
counts<-tapply(dr[,"Iswna"],INDEX=dr[,"Month"],FUN=sum)
se<-sdf/sqrt(counts)

# taking 95% confidence interval
qf<-qnorm(0.975)
```

```
# draping with the dot whiskers
for (i in 1:12) points(means[i],i,pch=8,cex=.5,col="red")
for (i in 1:12) lines(c(means[i]-qf*se[i],means[i]+qf*se[i]),
                      c(i,i),lwd=1,col="red",lty="solid")
for (i in 1:12) lines(rep(means[i]-se[i],2),c(i-0.07,i+0.07),
                      lwd=1,col="red",lty="solid")
for (i in 1:12) lines(rep(means[i]+se[i],2),c(i-0.07,i+0.07),
                      lwd=1,col="red",lty="solid")

# divide line separating total
lines(c(1,40),c(0.5,0.5),lwd=2,col="gray93",lty=1)

# calculating the mean and se for dot whiskers of total
dr$IswnaT<-!is.na(dr$wt)
meansT<-tapply(dr[,"wt"],INDEX=dr[,"IswnaT"],FUN=mean,na.rm=TRUE)
sdfT<-tapply(dr[,"wt"],INDEX=dr[,"IswnaT"],FUN=sd,na.rm=TRUE)
countsT<-tapply(dr[,"IswnaT"],INDEX=dr[,"IswnaT"],FUN=sum)
seT<-sdfT/sqrt(countsT)

# taking 95% confidence interval
qf<-qnorm(0.975)

# adding boxplot Total at 0 position on the active plot, add=TRUE
boxplot(dr$wt,data=dr,ylim=c(1,41),at=0,
        xlab="mass [g]",
        horizontal=TRUE,las=1,
        notch=T,boxwex=1.2,col="gray93",log="x",add = T)

# draping with the dot whiskers
points(meansT,0,pch=8,cex=.5,col="red")
lines(c(meansT-qf*seT,meansT+qf*seT),
      c(0,0),lwd=1,col="red",lty="solid")
lines(rep(meansT-seT,2),c(0-0.1,0+0.1),
      lwd=1,col="red",lty="solid")
lines(rep(meansT+seT,2),c(0-0.1,0+0.1),
      lwd=1,col="red",lty="solid")

# add ref line for mean
lines(c(meansT,meansT),c(0,12),lty=3,col="red")

# add ref line for median
med <- median(dr$wt) # calling the value
lines(c(med,med),c(0,12),lty=3)

# add axis label for total
axis(2,at=0,labels = "Total",las=1,font = 2)

# additional approximate terminating tick value
axis(1,40)
```

```
# Image at working directory, getwd()
dev.off()
getwd() # location where the image print as the name of the file
#########
par(mar=c(bottom=5,left=4,top=4,right=2)+0.1)#default lines
```

Appendix 5

Supplementary file – Fig_5_boxplotL.txt

```
# call file rest in working directory (wd)
dr <- read.csv("dario.csv")

# ordering the grouping sequences
dr$Month <- ordered(dr$Month,levels=c("Feb","Jan","Dec",
                                      "Nov","Oct","Sep",
                                      "Aug","Jul","Jun",
                                      "May","Apr","Mar"))

# openning the printing device to make image as .jpeg
png("Fig_5_boxplotL.jpeg",width = 5, height = 6,units = "in-
",res=588)

# changing default par settings
par(mar=c(bottom=3.9,left=3.9,top=1.3,right=1.3)+0.1)

# changing default font
par(family="serif")

# calculating the width ratio for boxplot
dr$Count<-dr$tl
dr$Count[dr$Count >= 0]<-1
dr$Count[is.na(dr$Count)== TRUE]<-0
sums<-tapply(dr[,"Count"],INDEX=dr[,"Month"],FUN=sum,na.rm=TRUE)
sums<-sums/max(sums)

# VARIABLE-WIDTH notched BOXPLOT call
boxplot(tl ~ Month,data=dr,ylim=c(4.5,13),xlim=c(0,12),
        xlab="total length [cm]", ylab="month",
        names=levels(dr$Month),horizontal=TRUE,las=1,
        width=sums,notch=T,boxwex=1.3,col="white")

# calculating the mean and se for dot whiskers
means<-tapply(dr[,"tl"],INDEX=dr[,"Month"],FUN=mean,
              na.rm=TRUE)
sdf<-tapply(dr[,"tl"],INDEX=dr[,"Month"],FUN=sd,na.rm=TRUE)
dr$Iswna<-!is.na(dr$tl)
```

```
counts<-tapply(dr[,"Iswna"],INDEX=dr[,"Month"],FUN=sum)
se<-sdf/sqrt(counts)

# taking 95% confidence interval
qf<-qnorm(0.975)

# draping with the dot whiskers
for (i in 1:12) points(means[i],i,pch=8,cex=.5,col="red")
for (i in 1:12) lines(c(means[i]-qf*se[i],means[i]+qf*se[i]),
                      c(i,i),lwd=1,col="red",lty="solid")
for (i in 1:12) lines(rep(means[i]-se[i],2),c(i-0.07,i+0.07),
                      lwd=1,col="red",lty="solid")
for (i in 1:12) lines(rep(means[i]+se[i],2),c(i-0.07,i+0.07),
                      lwd=1,col="red",lty="solid")

# divide line separating total
lines(c(4.5,13),c(0.5,0.5),lwd=2,col="gray93",lty=1)

# calculating the mean and se for dot whiskers of total
dr$IswnaT<-!is.na(dr$tl)
meansT<-tapply(dr[,"tl"],INDEX=dr[,"IswnaT"],FUN=mean,na.rm=TRUE)
sdfT<-tapply(dr[,"tl"],INDEX=dr[,"IswnaT"],FUN=sd,na.rm=TRUE)
countsT<-tapply(dr[,"IswnaT"],INDEX=dr[,"IswnaT"],FUN=sum)
seT<-sdfT/sqrt(countsT)

# taking 95% confidence interval
qf<-qnorm(0.975)

# adding boxplot Total at 0 position on the active plot, add=TRUE
boxplot(dr$tl,data=dr,ylim=c(4.5,13),at=0,
        xlab="total length [cm]",
        horizontal=TRUE,las=1,
        notch=T,boxwex=1.2,col="gray93",add = T)

# draping with the dot whiskers
points(meansT,0,pch=8,cex=.5,col="red")
lines(c(meansT-qf*seT,meansT+qf*seT),
      c(0,0),lwd=1,col="red",lty="solid")
lines(rep(meansT-seT,2),c(0-0.125,0+0.125),
      lwd=1,col="red",lty="solid")
lines(rep(meansT+seT,2),c(0-0.125,0+0.125),
      lwd=1,col="red",lty="solid")

# add ref line for mean
lines(c(meansT,meansT),c(0,12),lty=3,col="red")

# add ref line for median
med <- median(dr$tl) # calling the value
lines(c(med,med),c(0,12),lty=3)
```

```
# add axis label for total
axis(2,at=0,labels = "Total",las=1,font = 2)

# additional approximate starting and terminating tick value
axis(1,c(4.5,13),c("4.5","13"))

# Image at working directory, getwd()
dev.off()
getwd() # location where the image print as the name of the file
#########
par(mar=c(bottom=5,left=4,top=4,right=2)+0.1)#default lines
```

Table Appx 1. CSV file-dario.csv

Month	tl	wt
August	8.3	7.6
August	8.8	10.52
August	7.4	4.22
August	7.9	7.89
August	8.6	10.1
August	7.2	4.95
August	8.9	11.74
August	7.5	5.86
August	6.5	4.1
August	7.9	8.43
August	8.7	10.78
August	8.2	8.15
August	8.1	7.76
August	9.1	12.43
August	6.9	4.16
August	6.2	3.18
August	7.6	6.33
August	7.7	7.17
August	7.2	5.51
August	8.1	8.12
August	7	5.25
August	8.5	9.45
August	6.6	3.85
August	8	8.1

cont. Table Appx. 1

August	8.8	10.64
August	6.1	3.47
August	6.6	3.73
August	8.7	9.72
August	8.3	8.39
August	8.1	8.75
August	7.2	5.36
August	6.4	7.32
August	6.4	3.64
August	7.9	7.61
August	7.6	7.1
August	7.5	6.45
August	7.1	5.1
August	8.1	6.7
August	7.9	5.1
August	6.9	8.8
August	5.1	1.87
August	6.5	3.7
August	6.92	4.63
August	6.8	4.22
August	6.71	4.18
August	7.9	7.69
August	8.2	7.1
August	7.2	4.5
August	6.5	3.74
August	7.1	4.38
August	6.7	5.83
August	7.3	5.66
August	7.6	6.42
August	7.9	7.2
August	6.7	4.1
August	8.8	9.78
August	7.2	5.15
August	7.3	5.24
August	7.4	5.27
August	7.7	6.65

cont. Table Appx. 1

August	6.6	3.92
August	7.2	4.1
August	7.5	6.71
August	8.7	7.1
August	7.3	5.2
August	7.6	4.62
August	5.1	1.93
August	7.8	8.1
August	7.1	4.88
August	7	4.2
August	6.3	3.72
August	7.7	5.93
August	7.5	6.34
August	7.1	5.6
August	6.42	2.1
August	6.9	5.2
August	6.84	4.75
August	6.93	4.42
August	7.32	5.61
September	9.3	12.16
September	7.6	6.91
September	7.4	5.64
September	7.9	7.4
September	8.9	8.95
September	6.8	3.22
September	6	2.93
September	9.2	12.3
September	9.5	13.9
September	6.9	3.94
September	8.2	7.4
October	7.8	6.26
October	7.2	5.94
October	7.3	5.56
October	8.4	9.1
October	5	2.1
October	8.3	7.2

cont. Table Appx. 1

October	8.8	12.9
October	9	10.83
October	7.6	6.58
October	5.6	1.88
October	9.1	10.42
October	7.7	4.92
November	6.6	4.77
November	7.2	5.75
November	7.9	8.7
November	5.8	2
November	8.5	7.72
November	4.6	1.22
November	7.8	6.1
November	8.1	8.75
November	8	4.69
November	8.1	8.89
November	7.5	7.81
November	7.6	5.5
December	9.7	12.2
December	9.1	11.69
December	7.7	4.9
December	7.4	6.72
December	7.6	8.1
December	9.5	12.11
December	7.4	4.69
December	8.3	8.42
December	8.6	7.7
December	8.5	8.37
December	7.8	4.3
January	9.6	12.6
January	7.5	5.8
January	7.8	3.9
January	6.9	3.6
January	9.6	10.1
January	7	6.1
January	8.6	6.8

cont. Table Appx. 1

January	6.6	2.95
January	6.4	2.32
January	7.8	9.6
February	10.5	18.7
February	11.9	21.68
February	8.4	6.3
February	8.3	8.1
February	8.1	6.6
February	8.4	8.26
February	8.4	7.8
February	8.2	7.7
February	11.1	12.73
February	12.9	39.24
February	11.4	13.73
February	9.1	10.7
February	9.5	11.2
February	6.9	3.91
February	9.5	7.1
February	8.5	7.5
February	8.9	6.4
February	7.3	4.6
February	7.1	4.1
March	10.2	15.91
March	9.5	12.92
March	10.6	15.8
March	8.4	5.74
March	7.3	6.4
March	6.5	3.74
March	7.4	4.8
March	7.99	6.42
March	8.1	6.1
April	11.6	23.12
April	11.1	22.16
April	12.1	26.6
April	7.2	4.08
April	11.9	15.9

cont. Table Appx. 1

April	11.4	25.1
April	9.6	10.83
April	8.2	5.7
April	7.6	6.62
April	6.8	4.95
April	7.1	4.1
May	9.3	10.98
May	7.6	5.55
May	8.6	8.83
May	8.4	7.9
May	7.8	6.78
May	8.5	8.24
May	7.5	4.5
May	10.3	13.02
May	7.8	5.72
May	8.7	6.8
May	8.1	7.33
May	10.2	22.15
May	7.5	7.11
May	9.4	15.82
May	7.8	4.39
May	9.2	14.69
May	7.9	6.12
May	9.5	11.91
May	9.5	12.11
May	7.4	7.69
May	8.3	8.42
May	7.2	4.92
May	7.8	8.25
June	7.3	4.95
June	5.9	1.95
June	7.8	6.9
June	7.1	4.88
June	7	4.2
June	6.3	3.72
June	7.7	5.93

cont. Table Appx. 1

June	7.5	6.34
June	7.1	6.5
June	6.42	3.82
June	6.9	4.2
June	6.84	4.75
June	5.99	4.42
June	7.32	5.61
June	6.5	3.32
July	8.2	6.7
July	7.7	7.13
July	7	5.34
July	8.6	10.5
July	7.6	6
July	7.8	7.34
July	7.2	5.45
July	8.5	7.93
July	7.3	6.89
July	7.4	5.39
July	6.5	3.49
July	7.6	5.01
July	7.6	4.7
July	6.5	2.87

POLISH JOURNAL OF NATURAL SCIENCES
Abbrev.: Pol. J. Natur. Sc., Vol 37(2): 263–276, Y. 2022

DOI: 10.31648/pjns.7828

EVALUATION OF THE PROFILE AND CONTENT OF CHLOROPHYLL PIGMENTS AND ACIDITY IN SELECTED COLD PRESSED OILS

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Key words: chlorophyll pigments, cold pressed oils, acidity, pheophytinization, HPLC.

Abstract

The work evaluated the profile and content of chlorophyll pigments in cold pressed oils from rapeseed, flax, camelina, hemp, safflower, pumpkin, milk thistle, and from olive and avocado fruits, as well as the degree of hydrolysis of the oils examined. Content of chlorophylls *a* and *b* and their derivatives, being pheophytin *a*, pheophytin *b*, pyropheophytin *a* and pyropheophytin *b* were simultaneously determined in one sample, by modified and validated reversed phase HPLC method. The degree of hydrolysis of the oils was determined by their acid value. Market cold pressed oils have a very different content of chlorophyll pigments, regardless of whether they are from seeds or fruits. Considerable amounts were found in hemp oil (79.82 mg kg^{-1}) and pumpkin oil (57.55 mg kg^{-1}), and small quantity in flax oil (1.08 mg kg^{-1}).The content of chlorophyll pigments in extra virgin olive oil was an intermediate level (15.44 mg kg^{-1}). Chlorophyll derivatives dominate in the profile of chlorophyll pigments it is pheophytin *a*, pheophytin *b*, pyropheophytin *a* and pyropheophytin *b*. Extra virgin olive oil had the highest share of pheophytins, on average 82%. A statistically significant correlation (r = 0.6509) was found between the percentage share of pheophytins in the total content of chlorophyll pigments and the acid value of extra virgin olive oil. It was noted that the percentage share of pyropheophytins in the total of pheophytins and pyropheophytins can be an indicator of the bioconversion of chlorophylls during storage of oils. There was a statistically significant negative correlation (r = –0.8836) between the percentage share of pyropheophytins in the total of pheophytins and pyropheophytins and the length of the period remaining until the expiry date of extra virgin olive oil.

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Introduction

Chlorophylls are commonly green plant pigments. The major chlorophylls in plants include chlorophyll a and b, which occur approximate ratio 3 : 1 (SCHWARTZ and VON ELBE 1983). Chlorophyll pigments are natural components of oilseeds and fruits. It is known, that significant differences in contents of chlorophyll pigments in them occur depending on degree of maturity, which is determined by region of cultivation, form (winter or spring) and term and conditions of harvest (GANDUL-ROJAS et. al. 2016, ROCA et. al. 2016). Contents of chlorophyll pigments depend also on drying and storage conditions (KANAI et. al. 2010). They are included in all vegetable oils, in quantities depending on their content in raw material, pre-treatment parameters, pressing and purification (CRIADO et. al. 2007). The chlorophyll molecule consist of a central magnesium atom surrounded by a nitrogen-containing structure called a porphyrin ring; attached to the ring is a long carbon-hydrogen side chain known as a phytol. Chlorophyll is readily degraded when exposed to heat, light, oxygen, acids, and enzymes. Chlorophyll can degrade to undesirable grey-brown compounds such as pheophorbide and pheophytin. This degradation is mediated by acid and the enzyme chlorophyllase. Pheophorbide can be further metabolized to colorless compounds in metabolically active tissue. The effect of elevated temperature causes formation of pyro-compounds (HEATON and MARANGONI 1996, ROCA et. al. 2016). The hydrolytic, structural and thermal changes can have a significant influence on the content and composition of chlorophyll pigments (GANDUL-ROJAS et. al. 2016). Green color losses in processed and minimally processed fruit and vegetable products are associated with decrease in of quality of such products (HEATON and MARANGONI 1996). The presence of chlorophyll pigments in cold pressed oils has a negative effect on their taste, smell, color, clearness, thermal stability and shelf life. Those compounds are strong photosensitisers in oxidation processes of unsaturated bonds of lipids, that negatively impacts the oxidative stability of oil during storage (SYMONIUK et. al. 2018). Chlorophylls are photosensitizers that allow oxygen to transform into a singlet form that initiates the oxidation of unsaturated fatty acids (CHOE and MIN 2006). This is especially important when choosing the packaging and oil storage conditions (GARGOURI et. al. 2015). Cold pressed oils represent a small fragment of market food oils, but an increase in consumer interest in these products due to high nutritional properties has been observed (BRŰHL and MATTHÄUS 2006). They are obtained without changing the nature of the oil, by mechanical means, e.g. by expelling or pressing, without the use of heat. This category also includes virgin oils, i.e. oils and fats

obtained as a result of mechanical action and the possible use of thermal processing in the technological process. They can be cleaned only by applying water, sedimentation, filtration or centrifugation (*Codex Alimentarius* 2015). They are not refined, and therefore they are a rich source of antioxidants such as tocopherols, polyphenols and squalene (TUBEROSO et. al. 2007). These also contain polyunsaturated fatty acids from the n-3 and n-6 groups as well as sterols, which exert a bioactive effect (CHOO et. al. 2006, RACZYK et. al. 2016, RĘKAS et. al. 2016, TEH and BIRCH 2013). Chlorophylls and its derivatives play an important role in human nutrition, as anti-cancer factor, having antioxidant and anti-mutagenic activities (FERRUZZI and BLAKESLEE 2007). The content and profile of chlorophyll pigments plays an important role in oil stability (PSOMIADOU and TSIMIDOU 1998). Important parameter of cold-pressed oils is their acidity, which has significant influence on chlorophyll pigments profile (HEATON and MARANGONI 1996). Cold pressed oils were subject to numerous research, in which content of chlorophyll pigments was determined in total, after recalculation to pheophytin *a* (CHOO et. al. 2006, RACZYK et. al. 2016, RĘKAS et. al. 2016, SYMONIUK et. al. 2018, TEH and BIRCH 2013). In some research, knowledge of profile of chlorophyll pigments was used to determine quality and authenticity of olive oils (ANNIVA et. al. 2006, LAZZERINI et. al. 2016). For identification and quantitative determination of chlorophyll pigments mostly spectroscopic and high-performance liquid chromatography techniques were used (*AOCS Official Method* 2017, ISO 2014:AMD 1:2016, LAZZERINI et. al. 2016, PSOMIADOU and TSIMIDOU 1998).

Test materials were market oils popular among Polish consumers, that differed by term of shelf-life, acidity and content of chlorophyll pigments. It was taken into consideration that hydrolytic and structural changes that occur during their storage have significant influence on bioconversion of chlorophyll pigments. Hydrolytic changes and an increase in oil acidity have a direct impact on the pheophytinization of chlorophylls. Water in oil, involved in the hydrolysis of triacylglycerols, has only an indirect effect and therefore has not been determined. Chlorophylls are involved in the oxidation of vegetable oils in the presence of light. The kinetics of unsaturated lipids oxidation is influenced by a number of pro and antioxidant factors. Chlorophylls, in addition to metal ions such as Fe, Cu and enzymes, are an important pro oxidative factors. Oils also contain a number of other compounds with antioxidant activity, such as: tocopherols, sterols, phenolic compounds, squalene and corotenoids. The course of lipid oxidation process is also influenced by the type of packaging and storage conditions. It was found that with such multidirectional influence of various factors on the course of oxidation of market cold pressed oils, it will

be difficult to link these processes, measured only by the peroxide value, with the profile of chlorophylls. According to SYMONIUK et. al. (2018) total chlorophylls content is only weak correlated with the induction time measured in the Rancimat test.

The purpose of the work was evaluation of the profile and content of chlorophyll pigments and acidity in selected cold pressed oils. The work was also aimed at determining the relationship between profile of chlorophyll pigments and the degree of hydrolysis of oil and the time till the end of shelf-life.

Materials and Methods

Materials

The test materials were oils, from seeds and fruits, delivered to the retail network by various producers. The oils were cold pressed according to declaration of producers. Oils were purchased in one of Warsaw's supermarkets. The oils came from the seeds of: rapeseed, flax, camelina, hemp, safflower, pumpkin, milk thistle and from olives and avocado fruits. In total, 45 oil samples were tested. All oils were in their shelf-life. The period from testing time to the end of the shelf-life was determined basing on the dates printed on the packaging. The oils were packed in glass bottles, light, brown or green, with a capacity from 0.25 to 1 dm^3 (Tab. 1). Chemical analyzes were performed within 10 days of purchase of oils. During analysis the samples of oils were stored in freezing conditions.

Table 1

Kind of oil and their origin, volume and color of packaging and periods up to the end of shelf-life

Kind of oil	No.	Brand name / Country of origin	Color of glass of bottle	Bottle volume [dm^3]	Best for period [month]
1	2	3	4	5	6
Rapeseed	1	Oleofarm / Poland	brown	0.5	6
	2	Semco / Poland	brown	0.25	6
	3	Kruszwica / Poland	green	0.5	8
	4	Olvita / Poland	clear	0.5	10
	5	Olandia / Poland	clear	0.5	10
Flax	1	Semco / Poland	green	0.25	3
	2	Kruszwica / Poland	green	0.25	1
	3	Eurolen / Poland	brown	0.5	2

cont. Table 1

Camelina	1	Semco / Poland	brown	0.25	4
	2	Olvita / Poland	brown	0.25	4
	3	Olandia / Poland	green	0.25	3
Hemp	1	Oleofarm / Poland	brown	0.25	9
	2	Olvita / Poland	brown	0.5	8
	3	Olini / Poland	brown	0.25	10
Safflower	1	Oleofarm / Poland	brown	0.25	4
	2	Olvita / Poland	clear	0.25	8
	3	Perlo / Poland	brown	0.5	6
Pumpkin	1	Oleofarm / Poland	brown	0.25	9
	2	Kruszwica / Poland	green	0.25	8
	3	Monini / Italy	green	0.25	6
Milk thistle	1	Oleofarm / Poland	brown	0.25	2
	2	VitaCorn / Poland	green	0.25	5
	3	Herbanordpol / Poland	brown	0.5	4
Olive extra virgin	1	Carras Queno/ Spain	clear	0.25	2
	2	Los Raigones / Spain	brown	0.5	15
	3	OlivaOro / Spain	brown	0.5	5
	4	Carrefour / Tunisia	green	0.25	10
	5	Goccia D˙oro / Italy	clear	1	12
	6	La Espanola / Spain	clear	0.75	5
	7	La laguna / Spain	green	0.5	17
	8	Carras Queno / Spain	clear	0.5	12
	9	Pons / Spain	green	0.5	17
	10	Cirio / Italy	brown	1	13
	11	Melissa Primo Gusta / Greece	brown	0.5	21
	12	Goccia D˙oro / Italy	clear	1	19
	13	Olivari / Portugal	green	0.5	30
	14	Olitalia / Italy	green	0.5	12
	15	Ondoliva / Spain	green	1	10
	16	Monini Delicato / Italy	green	1	19
	17	Monini Classico / Italy	green	0.5	31
	18	Salvadori / Italy	clear	0.5	12
	19	Di Carlo / Italy	clear	0.5	9
Avocado	1	Olvita / Kenya	brown	0.25	5
	2	Frontier Foods / Mexico	green	0.25	5
	3	Oleofarm / South Afrika	brown	0.25	7

Methods

Content of chlorophylls a and b and their derivatives, it is pheophytin *a*, pheophytin *b*, pyropheophytin *a* and pyropheophytin *b* were simultaneously determined in one sample, by modified and validated reversed phase HPLC method (ISO 29841:2014/AMD 1:2016) The analysis of chlorophyll pigments was carried out with using reference substances. Both chlorophylls: *a* (chl *a*) and *b* (chl *b*) were purchased from Sigma Aldrich (Poznan, Poland). Pheophytin *a* (phy *a*) and pheophytin *b* (phy *b*) were prepared from corresponding chlorophylls by acidification ether solutions with 13% hydrochloric acid (SCHWARTZ and VON ELBE 1983). Pyropheophytin *a* (pyr *a*) and pyropheophytin *b* (pyr *b*) were prepared from corresponding pheophytins dissolved in pyridine, by heat treatment at 110°C (PENNINGTON et. al. 1964). The HPLC-grade solvents, methanol and acetone, were obtained from Avantor (Gliwice, Poland), and diethyl ether from Sigma Aldrich (Poznan, Poland). De-ionized water was made using a Milli-Q purification system from Millipore (Bedford, MA, USA). Other chemicals were of analytical-reagent grade and were used without further purification. Hydrochloric acid (purity ≥ 37%) and sodium sulfate anhydrous (purity ≥ 99.0%) were purchased from Avantor (Gliwice, Poland) and pyridine anhydrous (purity ≥ 99.8%) were obtained from Sigma Aldrich (Poznan, Poland). Mobile phases were filtered through a Millipore 0.22 μm membrane filter before usage. An HPLC system (Agilent Technologies Series 1100, Santa Clara, CA, USA) composed of a G-1379A vacuum degasser, a G-1311A quaternary pump, a G-1313A autosampler, a G-1316A column oven and G-1321A fluorescence detector. Agilent Chemstation for LC and LC/MS systems software was used. Vortex mixer was from JWE-electronic, Warsaw, Poland. The C18 column Aeris PEPTIDE XB (3.6 μm, 250 mm length x 4.6 mm ID.) were from Phenomenex Corp. (Torrance, CA, USA). FLD detector (excitation wavelength λ_{ex} = 430 nm and emission wavelength λ_{em} = 670 nm) was employed, and program elution with 0.8 ml/min flow rate at temperature 25°C was used. The injection volume was 20 μL. The mobile phase was a gradient prepared from water : methanol : acetone 4 : 76 : 20 (solvent A) and methanol : acetone 30 : 70 (solvent B). Initial conditions: 100% A by 3 min, next decrease of share of A to 0% by 10 min, holding that condition by 18 min, after which increase share A to 100% by 22 min and stabilization of system in initial conditions by 30 min. Identification of chlorophylls and derivatives was carried out on the basis of spectral analysis using the UV-VIS spectrophotometer at wavelengths from 400 to 700 nm. Calibration curves were prepared using calibration standard solutions. Limits of detection (LOO_s) and limits of quan-

tification (LOQ_s) were determined. Content of chlorophylls and derivatives was calculated, using equations of standard curves, experimentally determined for examined ranges of concentrations. 2 g of oil was weighed into a glass tube and acetone was added to volume of 10 ml. The sample was vortex mixed for 1 min.

The acid value (AV) of oils was determined according to the ISO 660:2010.

Statistical analysis. All analyses were made in triplicates. Statistical analysis was performed using Statistica 13, TIBCO Software Inc. (2017). Evaluation of significance of deviations of average contents of chlorophyll pigments in oils was done using one way analysis of variance and Tukey`s post hoc tests. Correlation coefficients were also determined, to show the relations between percentage share of pheophytins, in total content of chlorophyll pigments and acid value, and between percentage share of pyropheophytins in sum of pheophytins and pyropheophytins and the amount of time till the expiry date. All tests were done assuming significance level of 0.05.

Results and Discussion

The market characteristics of the oils are given in Table 1. It shows that all cold pressed oils were packed in glass bottles. More than 20% of the tested oils were improperly packaged in clear glass bottles. In oils packed in clear glass, due to the access of light, accelerated oxidation takes place initiated by photochemical reactions catalyzed by chlorophyll pigments (Roca et. al. 2016). Unfavourable changes can be reduced by using the right type of packaging, such as dark glass bottles and metal cans or as well as by reducing the air content in the packaging by blowing inert gases and forming a cushion of these gases above the oil surface (Gargouri et. al. 2015, Wroniak et. al. 2016). The cold-pressed oils were characterized by very different shelf life declared by producers – from seeds between 3 (flax) and 24 months (safflower) and from fruits between 12 (avocado) and 36 months (olive extra virgin). Time periods from date of analysis up to the end of shelf-life products, differs – from seeds, between 1 and 10 months and from fruits, between 2 and 31 months (Tab. 1, col. 6). This could have some influence on the results obtained (Garcia-González et. al. 2008, Vujasinovic et. al. 2010). The results of our research are presented in Table 2 (for seeds), and 3 (for fruits). There was a significant variation in the content of chlorophyll pigments depending on the kind of oil.

Table 2

Content of chlorophyll pigments and acid value of oils derived from seeds

Kind of oil	No.	Content of chlorophyll pigments [mg kg^{-1}]							AV [mg KOH g^{-1}]
		chl *a*	phy *a*	pyr *a*	chl *b*	phy *b*	pyr *b*	total	
Rapeseed	1	0.73	0.32	0.24	n.d.	0.09	0.06	1.44	1.05
	2	0.41	1.76	0.31	n.d.	0.18	0.22	2.88	2.57
	3	0.59	0.12	n.d.	n.d.	0.06	0.02	0.79	1.12
	4	0.46	0.70	0,26	n.d.	0.12	0.14	1.68	1.33
	5	0.55	1.95	0.34	n.d.	0.20	0.28	3.32	2.89
	$\bar{x}$ ± SD	0.55^{a} ± 0.12	0.97^{a} ± 0.84	0.23^{a}± 0.12	n.d.	0.13^{a}± 0.06	0.14^{a}± 0.11	2.02^{a}± 1.05	1.79 ±0.87
Flax	1	0.49	0.11	n.d.	n.d.	0.07	nd.	0.67	1.01
	2	0.44	0.05	n.d.	n.d.	0.05	n.d.	0.54	0.97
	3	0.47	1.19	n.d.	n.d.	0.27	0.11	2.04	1.94
	$\bar{x}$ ± SD	0.47^{a}± 0.03	0.45^{a}± 0.03	n.d.	n.d.	0.13^{a}± 0.12	0.04^{a}± 0.05	1.08^{a}± 0.83	1.31± 0.55
Camelina	1	0.51	2.51	0.27	n.d.	0.46	0.50	4.25	2.05
	2	0.48	0.60	0.37	n.d.	0.18	0.15	1.78	1.27
	3	0.26	1.37	0.36	n.d.	0.27	0.20	2.46	1.46
	$\bar{x}$ ± SD	0.42^{a}± 0.14	1.49^{a}± 0.14	0.33^{a}± 0.06	n.d.	0.30^{a}± 0.14	0.28^{a}± 0.19	2.83^{a} ± 1.26	1.63 ±0.45
Hemp	1	0.69	38.02	3.89	3.02	9.68	7.78	63.08	3.01
	2	0.88	61.54	6.82	n.d.	25.98	15.17	110.39	3.59
	3	0.99	41.17	5.87	2,19	8.78	6.98	65.98	2.26
	$\bar{x}$ ± SD	0.85^{a}± 0.15	46.91^{b}± 12.77	5.53^{c} ± 1.49	1.74± 1.27	14.81^{b}± 9.68	9.98^{b}± 4.52	79.82^{b}± 26.52	2.95 ±0.67
Safflower	1	0.44	0.14	0.21	n.d.	0.09	0.03	0.91	1.15
	2	0.49	0.11	0.42	n.d.	0.19	0.28	1.49	1.22
	3	0.20	0.24	0.18	n.d.	0.15	0.05	0.82	1.11
	$\bar{x}$ ± SD	0.38^{a}± 0.16	0.16^{a}± 0.07	0.27^{a}± 0.13	n.d.	0.14^{a}± 0.05	0.12^{a}± 0.14	1.07^{a}± 0.36	1.16 ± 0.06
Pumpkin	1	17.94	12.98	1.77	n.d.	31.63	8.76	73.08	1.17
	2	10.23	6.99	2.11	n.d.	23.82	4.01	47.16	3.31
	3	16.01	7.98	2.18	n.d.	21.00	5.23	52.40	2.01
	$\bar{x}$ ± SD	14.73^{b}± 4.01	9.32^{a}± 3.21	2.02^{b}± 0.22	n.d.	25.48^{b}± 5.51	6.00^{b}± 2.47	57.55^{b}± 13.71	2.16 ±1.08
Milk thistle	1	0.50	0.94	0.39	n.d.	0.41	0.23	2.47	1.27
	2	0.44	0.89	0.28	n.d.	0.30	0.11	2.02	1.04
	3	0.31	1.15	0.27	n.d.	0.27	0.15	2.15	1.34
	$\bar{x}$ ± SD	0.42^{a}± 0.10	0.99^{a}± 0.14	0.31^{a}± 0.07	n.d.	0.33^{a}± 0.07	0.16^{a}± 0.06	2.21^{a} ± 0.23	1.22 ±0.16

Values (means ± SD) bearing different superscripts are statistically significantly different ($p < 0.05$); n.d. – not detected; chl *a* – chlorophyll *a*; phy *a* – pheophytin *a*; pyr *a* – pyropheophytin *a*; chl *b* – chlorophyll *b*; phy *b* – pheophytin *b*; pyr *b* – pyropheophytin *b*; AV – acid value

The most chlorophyll pigments in oils from seeds contained hemp oil of an average of 79.82 mg kg^{-1}. These are amounts typical for oil pressed from mature seeds (TEH and BIRCH 2013*)*. The rich source of chlorophyll pigments was also pumpkin seed oil (an average of 57.55 mg kg^{-1}). It is clearly higher than data earlier presented (SYMONIUK et. al. 2018). Considerably less chlorophyll pigments were found in other oils. The total content of chlorophyll pigments in rapeseed oil was on average 2.02 mg kg^{-1} (from 0.79 to 3.32 mg kg^{-1}), which is in agreement with previously published data (GHAZANI et. al. 2014, RĘKAS et. al. 2016). The higher level of chlorophyll pigments in cold pressed rapeseed oil is presented by YANG et. al. (2013). Content of these compounds in flax oil was on average 1.08 mg kg^{-1} (range from 0.54 to 2.04 mg kg^{-1}). These quantities do not differ from the literature data (CHOO et. al. 2006, RACZYK et. al. 2016) but are clearly smaller than other presented by TEH and BIRCH (2013). The content of chlorophyll pigments in camelina oil ranged from 1.78 to 4.25 mg kg^{-1}. Smaller variability was presented by SYMONIUK et. al. (2018). The content of chlorophylls in milk thistle oil was on average 2.21 mg kg^{-1} and do not differ from the literature data (MALEKZADEH et. al. 2011). In extra virgin olive oil, the average content of chlorophyll pigments was 15.44 mg kg^{-1} (variability from 2.16 to 37.94 mg kg^{-1}). These are the content typical for this product (GANDUL-ROJAS et. al. 2016). Avocado oil contained significant amounts of chlorophyll pigments (average 73.56 mg kg^{-1}), many times exceeding the literature data (WONG et. al. 2010). This can be caused by the immaturity of the fruits and technological process. The process for recovering oil from avocados is mechanical extraction, similar to olive oil extraction, with the additional step of removing the skin. Skin is abundant source of chlorophylls and when the technological process has been incorrectly carried out it remains in the raw material subjected to cold pressing (WONG et. al. 2011). Chlorophylls a and b were a small fragment of a pool of chlorophyll pigments in analyzed oils. The proportion of chlorophyll a varied from 0.5% in olive oil to 27.2% in rapeseed oil. A significant, unusual share of chlorophyll a was in flax oil (43.5%) may be due to seed immaturity. Chlorophyll b was absent in rapeseed, flax, camelina, milk thistle and safflower oils. The proportion of chlorophyll *b* in other oils ranged from under 0.1% in avocado oil to 2.2% in hemp oil. The lack or small content of chlorophylls *a* and *b* in oils indicates their profound changes to derivatives that took place from the raw material to the point of analysis. The oils were dominated by pheophytins a and b. Share of pheophytins in total content of chlorophyll pigments varied from 53.7% (flax oil) to 77.3% (hemp oil). The exception was safflower oil, in which deep bioconversion of pheophytins to pyropheophytins occurred. Share of phe-

ophytins in total content of chlorophyll pigments was high in fruit oils, 72.9% in avocado oil and 81.5% in extra virgin olive oil. In case of extra virgin olive oil, the significant amount of pheophytins is typical for technological process applied. The fruits of the olives after crumbling are subjected to malaxation, i.e. slow mixing of the oil paste with the addition of warm water. During malaxation, in the presence of organic acids from the fruits, at reduced pH, the chlorophylls are pheophytinized, i.e. the process bioconversion in the corresponding pheophytins (CRIADO et. al. 2007). A similar process can also take place in seeds with high water content, if they are stored for too long before drying. Then the hydrolysis of triacylglycerols and the increase in the content of FFAs took place, which promotes the conversion of chlorophylls to pheophytins (GARCIA-GONZÁLEZ et. al. 2014). Other derivatives of chlorophylls found in the oils were pyropheophytins, a and b. Pyropheophytins criterion showed good performance as indicator of overall quality and freshness of oils as well as highlighting any problems during the storage of the product (GUILLAUME et. al. 2014). The percentage of pyropheophytins in the total content of pheophytins and pyropheophytins can be a useful indicator of these undesirable changes. In the range of 0–20%, considered appropriate by ANNIVA et al. (2006), there were oils: rapeseed, flax, hemp, pumpkin and olive extra virgin. In the range of 20–30%, oil was found to be excessive: camelina, milk thistle and avocado. Safflower oil was characterized by a very high proportion of pyroforms ranging from nearly 50% to almost 90%. Basing on data from Table 1 and Table 3 (for 17 out of 19 samples) it was stated that statistically significant, negative correlation exists between % share of pyropheophytins in sum of pheophytins and pyropheophytins (y) and the amount of time till the expiry date of extra virgin olive oil (x) ($r = -0.8836$), with the following equation of regression:

$$y = -0.525x + 26.34.$$

Thus, during storage of extra virgin olive oil pheophytins are converted into pyropheophytins and the closer to the expiry date there are more pyro-derivatives. Correlation of $r = -0.8836$ suggests a strong, negative relationship between studied variables. Degradation of pheophytins to pyropheophytins in olive oil is visible already after 3 months of storage (GALLARDO-GUERRERO et. al. 2005). Seeds oils showed various acid value varying from 1.16 mg KOH g^{-1} for safflower oil to 2.95 mg KOH g^{-1} for pumpkin oil. In any case acid value did not exceed acceptable value of 4 mg KOH g^{-1} (*Codex Alimentarius* 2015). Similar results were also reported (TEH and BIRCH 2013, YANG et. al. 2013). During storage of oil, as a result of progressive hydrolysis (enzymes, traces of water) its acidity grows,

which stimulates bioconversion of chlorophylls to pheophytins (VUJASINOVIC et. al. 2010, YANG et. al. 2013). Basing on data from Table 2 a correlation was determined ($r = 0.7379$) between percentage share of pheophytins in total content of chlorophyll pigments (y) and acid value of seeds oils (x), with the following equation of regression:

$$y = 17.187x + 26.71.$$

Table 3

Content of chlorophyll pigments and acid value of oils derived from fruits

Kind of oil	No.	Content of chlorophyll pigments [mg kg^{-1}]							AV [mg KOH g^{-1}]
		chl *a*	phy *a*	pyr *a*	chl *b*	phy *b*	pyr *b*	total	
Olive extra virgin	1	0.46	2.99	3.76	n.d.	0.30	1.19	8.70	0.89
	2	n.d.	27.60	5.36	n.d.	2.90	2.08	37.94	1.82
	3	n.d.	1.25	0.62	n.d.	0.07	0.22	2.16	0.71
	4	0.49	5.37	0.82	n.d.	0.43	1.16	8.27	0.77
	5	n.d.	9.50	1.29	n.d.	0.37	1.76	12.92	1.01
	6	n.d.	4.14	1.29	n.d.	0.37	0.90	6.70	0.94
	7	0.44	12.29	2.01	n.d.	0.61	1.12	16.47	1.44
	8	n.d.	17.45	3.82	n.d.	1.48	1.34	24.09	1.54
	9	n.d.	9.06	1.52	n.d.	0.73	0.85	12.16	1.04
	10	n.d.	9.00	1.65	n.d.	0.80	1.15	12.60	1.11
	11	n.d.	17.10	0.30	2.45	0.60	1.70	22.15	1.39
	12	n.d.	15.10	0.25	1.05	0.60	1.70	18.70	1.29
	13	n.d.	22.90	0.60	0.75	1.80	1.25	27.30	1.52
	14	n.d.	9.75	1.70	n.d.	0.60	1.25	13.30	1.13
	15	n.d.	6.70	1.55	n.d.	0.55	0.90	9.70	0.67
	16	n.d.	16.75	0.30	n.d.	0.65	1.75	19.45	1.23
	17	n.d.	18.85	0.25	n.d.	0.80	1.40	21.30	1.40
	18	n.d.	12.65	n.d.	n.d.	0.70	n.d.	13.35	1.18
	19	n.d.	6.05	n.d.	n.d.	n.d.	n.d.	6.05	0.65
	$\bar{x}$ ± SD	0.07± 0.17	11.82± 6.85	1.43± 1.42	0.22± 0.59	0.76 ±0.65	1.14± 0.65	15.44± 8.38	1.14 ±0.32
Avocado	1	1.02	61.49	5.92	n.d.	25.21	13.13	106.77	0.98
	2	1.26	18.61	12.66	0.03	5.03	8.65	46.24	0.76
	3	0.18	33.21	7.31	0.07	17.22	9.67	67.66	0.80
	$\bar{x}$ ± SD	0.81± 0.46	37.77± 17.80	8.63± 2.91	0.03± 0.03	15.82± 8.30	10.48± 1.91	73.56± 25.06	0.85 ±0.10

Values (means±SD); n.d. – not detected; chl *a* – chlorophyll *a*; phy *a* – pheophytin *a*; pyr *a* – pyropheophytin *a*; chl b – chlorophyll *b*; phy *b* – pheophytin *b*; pyr *b* – pyropheophytin *b*; AV – acid value

This correlation was not statistically significant though, with significance level of 0.05. The lack of significant correlation between the studied variables could result from several reasons. The oils came probably from seeds of varying quality and were probably stored under different conditions. As it can be assumed, it influenced their acidity. The differences in the shelf life of oils and the color of the glass bottle indicate different rates of lipid oxidation, which could also contribute to changes in the profile of chlorophyll pigments. Acidity of oils coming from fruits was lower than seeds oils. Average acid value of extra virgin olive oil was 1.14 mg KOH g^{-1} (range from 0.65 to 1.82 mg KOH g^{-1}) and avocado oil 0.85 mg KOH g^{-1} (range from 0.76 to 0.98 mg KOH g^{-1}). Basing on data from Table 3 a correlation was determined ($r = 0.6509$) between percentage share of pheophytins in total content of chlorophyll pigments (y) and acid value of extra virgin olive oil (x), with the following equation of regression:

$$y = 16.421x + 59.22.$$

It was confirmed that higher acidity fosters pheophytinization of chlorophylls. Correlation of $r = 0.6509$ suggests a moderate, positive relationship between studied variables. The obtained results could also be influenced by the differences in the technologies used. In the case of seeds, after cleaning, they are directly pressed on a screw press. In the process of obtaining extra virgin olive oil, an additional malaxation process is used, in which chlorophylls largely convert into the appropriate pheophytins before pressing.

Conclusions

Market cold pressed oils have a very different content of chlorophyll pigments, regardless of whether they are from seeds or fruits. Considerable amounts were found in hemp oil (79.82 mg kg^{-1}) and pumpkin oil (57.55 mg kg^{-1}), and small quantity in flax oil (1.08 mg kg^{-1}). The content of chlorophyll pigments in extra virgin olive oil was an intermediate level (15.44 mg kg^{-1}). Chlorophyll derivatives dominate in the profile of chlorophyll pigments i.e. pheophytin *a*, pheophytin *b*, pyropheophytin *a* and pyropheophytin *b*. Extra virgin olive oil had the highest share of pheophytins, on average 82%. The acidity of extra virgin olive oil influences the profile chlorophyll pigments. Statistically significant correlation ($r = 0.6509$) was observed, between percentage share of pheophytins in total content of chlorophyll pigments and acid value of extra virgin olive oil. The percentage of pyropheophytins in the total content of pheophytins

and pyropheophytins can be an indicator of the transformation of chlorophylls during storage of extra virgin olive oil. Statistically significant negative correlation ($r = -0.8836$) exists, between percentage share of pheophytins in sum of pheophytins and pyropheophytins and length of the period which was left till the expiry date of extra virgin olive oil.

Translated by MONIKA BARTOSIAK

Accepted for print 24.05.2022

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