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INDICES IN THE EVALUATION OF THE FUNCTIONAL ACTIVITY OF BLOOD CELLS OF THE BOTTLENOSE DOLPHIN *TURSIOPS TRUNCATUS* (MONTAGU, 1821)

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The content of cationic protein in granulocytes of the bottlenose dolphin *Tursiops truncatus* (Montagu, 1821) was established by calculating the average cytochemical coefficient. Its shortcomings were substantiated in visually determining the intensity of staining of the product of a cytochemical reaction on blood products and distributing cells into groups according to the amount of protein they contain. To assess the activity of a substance in a cell, computer programs were applied, and a light microscope was used which allows to minimize errors in morphometric measurements of objects. Individual parameters were calculated for the degree of filling and intensity of staining of cationic protein in granulocytes in bottlenose dolphins with and without taking into account the protein content in the entire blood volume. Such indicators allow carrying out comparative age-related, intraspecific, and interspecific studies in animals. As established, the content of cationic protein in granulocytes can vary greatly in different individuals of bottlenose dolphins, and its amount changes slightly with age.

Keywords: morphometry, average cytochemical coefficient, integral cytochemical index, cationic protein, granulocytes, bottlenose dolphin

In Russia, marine mammals are kept in oceanariums and dolphinariums mainly for commercial purposes. Only few of such organizations carry out research to assess health of pinnipeds and cetaceans and their immune status [Andreeva et al., 2013; Derko et al., 2018; Duvanova, Denisenko, 2018; Kaganova, 2018; Lauderdale et al., 2021; Romanov et al., 2023; Semenov et al., 2020; Vasileva, 2019; Zakharenko, 2019]. Clinical, biochemical, and cytomorphological blood tests are widely used to determine the functional health of marine mammals. To date, a large number of such studies have been carried out applying hematological and biochemical analyzers, flow cytometers, *etc.* [CRC Handbook, 2018; Keogh et al., 2011; Lauderdale et al., 2021; Nouri-Shirazi et al., 2017; Tryland et al., 2006]. Sampling of blood from animals in their natural habitat and its delivery are often limited by the distance from laboratories and acceptable storage periods for biomaterial. Blood smears from animals, both in captivity and in the wild, can be stored for a long time and allow describing and assessing the health of mammals later, with modern equipment used.

Precise mathematical measurements of cellular structures using computer morphometry allow carrying out comparative age, intraspecific, and interspecies studies of animals. Quantitative and qualitative techniques are effective in establishing the functional activity of mammalian blood cells. Quantitative analysis is aimed at identifying individual cell groups and determining their ratio (*e. g.*, white blood cell count), estimating the number of granules and nuclear elements in individual cells, and measuring areas of a substance stained by various cytochemical reactions. Qualitative analysis allows registering the intensity of staining of the cytochemical reaction product. Each of these techniques involves the use of dimensional (length, width, radius, area, and so on) and quantitative (number of granules and segments) characteristics of the objects investigated.

Qualitative analysis requires determining not only dimensional characteristics, but also color ones (*e. g.*, optical density) of the reaction product. Such an assessment of the functional activity of a cell involves the use of special equipment. At the same time, accurate dimensional and color characteristics do not depend on subjectivity of a researcher and their experience. When determining morphometric indices of cells on blood smears, absolute and relative parameters are used. Absolute ones are cell area, its diameter, and its shape, as well as number of granules and cellular elements in the cell. To establish these parameters, in each smear, cells are measured which are freely located in the visible field without overlapping and deformation from nearby cells: this allows to exclude their compression depending on the smear density. The use of relative parameters helps in recording characteristics of cellular structures regardless of the smear density.

Quantitative characteristics are simple and convenient for determining the functional activity of cells, but those provide a subjective and only general assessment of the intensity of cellular processes. A semi-quantitative method is widely used: the calculation of the average cytochemical coefficient [Letsky, 1973] which allows estimating the average cytochemical activity of a living organism based on the distribution pattern of the stained substance in a cell. White blood cell count is effectively applied; based on it, leukocyte indices are determined [Davis et al., 2008; Garkavi et al., 1990; Kal'f-Kalif, 1941; Mustafina et al., 1999; Ostrovsky et al., 2006, 2007; Speransky et al., 2009]. The comprehensive use of hematological indices provides a large amount of information and helps in assessing development, severity, and course of an inflammatory process and endogenous intoxication and in analyzing the general immunological reactivity of an organism. Application of these coefficients and indices is informative both separately and together. At the same time, some quantitative indicators are not perfect.

The aim of this work is to study the possibility of using additional coefficients and indices to assess the functional activity of proteins and enzymes in the bottlenose dolphin blood cells on the example of cationic protein in granulocytes.

MATERIAL AND METHODS

The object of the study are dolphins *Tursiops truncatus* (Montagu, 1821) aged 1 to 16 years. Material from 14 individuals was obtained in the Sevastopol oceanarium. Blood was sampled from caudal veins of bottlenose dolphins. Blood smears were prepared in accordance with a generally accepted technique and fixed in methanol for 5 min before staining. The preparations were stained with fast green after M. Alfert and I. Geschwind [Butenko et al., 1974] and investigated using oil immersion under an Axio Imager M1 microscope equipped with an AxioCam digital video camera and AxioVision software for analyzing images of microobjects (manufactured by Zeiss). To register the content of cationic

protein (hereinafter CP), the average cytochemical coefficient was calculated [Letsky, 1973], the cell area was determined, and area and optical density of the cytochemical reaction product were recorded. Two cytochemical characteristics were established: cell filling index (hereinafter CFI) and integral cytochemical index (hereinafter ICI) [Slavinsky, 2000].

RESULTS AND DISCUSSION

To calculate the average cytochemical coefficient (hereinafter ACC), the degree of reaction intensity is determined visually by the amount of the stained substance in the cell cytoplasm (Fig. 1). Granulocytes are divided into groups: 0 (no staining or granules in the cytoplasm); A, low-active cells (the occurrence of single granules or staining); B, moderately active (the studied substance fills almost the entire cell in leukocytes, but unstained areas of the cytoplasm may remain); and C, highly active (intensively stained granules [substance] fill the entire cytoplasm). ACC is calculated by the formula ACC = (3C + 2B + A) / 200. In each smear, 200 granulocytes were taken into account.

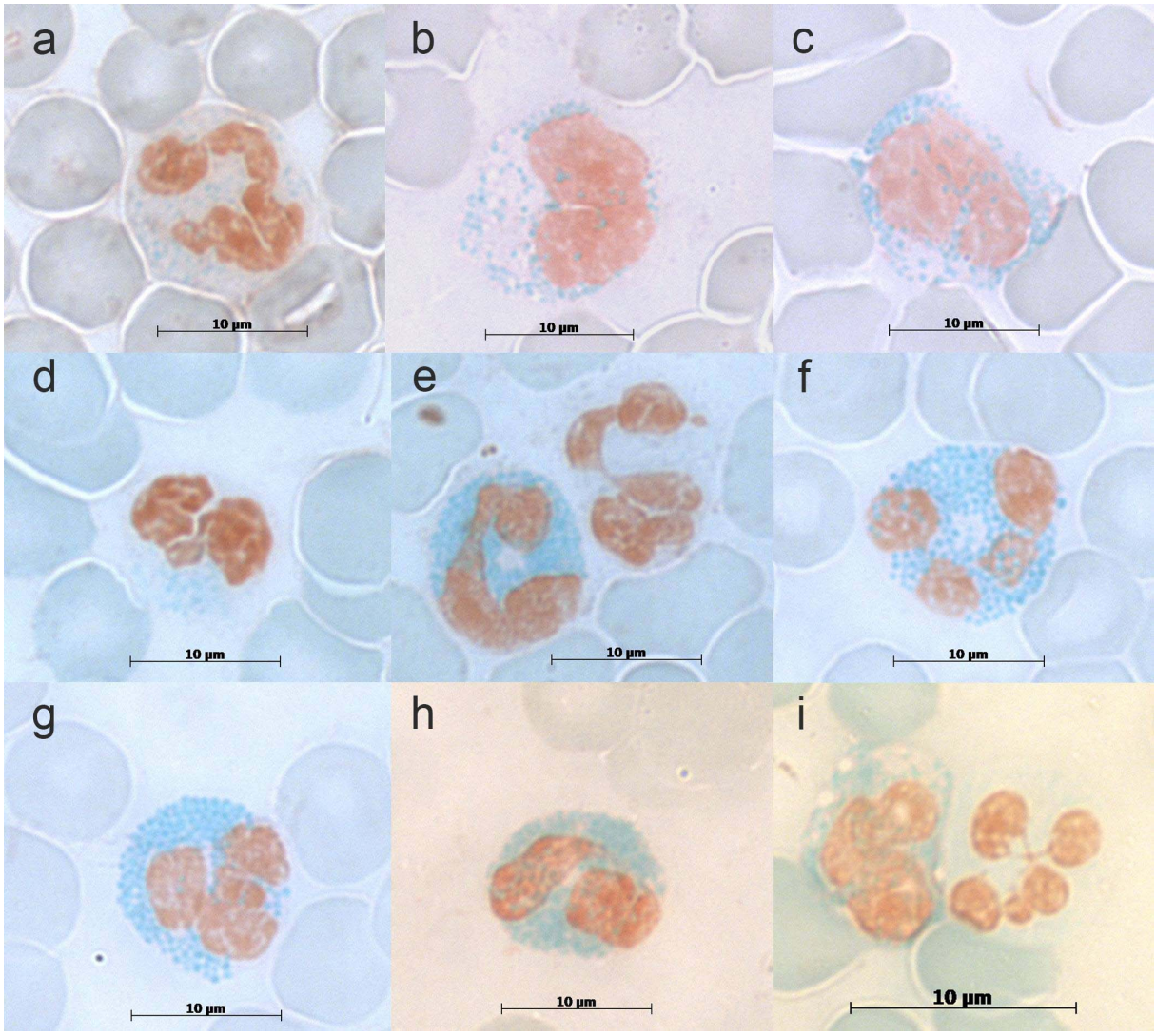

Fig. 1. Bottlenose dolphin granulocytes. Staining for cationic protein after M. Alfert and I. Geschwind [Butenko et al., 1974] (see text for explanation)

Visually, it is hard to distribute cells into the above-described groups, especially to assign them to categories A and B. Low-active cells with single granules (A) can include both a cell with one granule and a cell with a small area of the stained substance filling a quarter of a cell or less (Fig. 1a, b, d). Moderately active granulocytes (B) are also represented by a wide range of patterns: from a third of the stained substance in a cell (Fig. 1c, i) to almost complete filling with granules or the active substance (Fig. 1e, f, g, h). When distributing cells into groups, highly active granulocytes (C) can be the ones where the active substance occupies almost the entire cell, with only small areas being free of granules or the stained substance (Fig. 1g, h); however, according to the classification, this type of staining belongs to group B.

To reduce the share of stained cells mistakenly assigned to a particular group, we used additional parameters for assessing the activity of the substance. We determined cell area, optical density, and area of the cytochemical reaction product (Fig. 2). CFI and ICI were calculated [Slavinsky, 2000]. CFI is the share of the total area of the structures measured (stained CP granules) in the cell area (Fig. 2a, b). ICI is the product of the total area of the cytochemical reaction product in the cell and its optical density corresponding to the amount of stained CP (Fig. 2b).

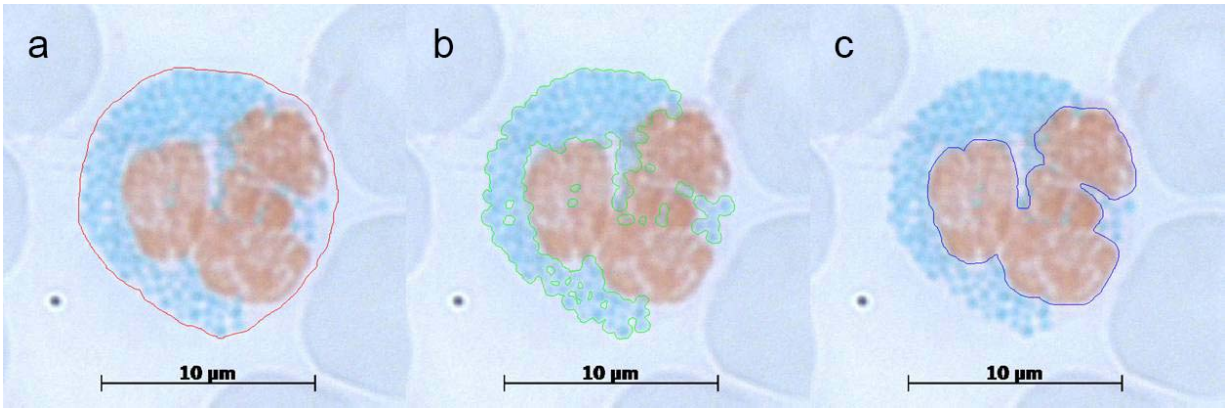

Fig. 2. Bottlenose dolphin granulocytes: a, the structures of the entire cell are highlighted; b, the area of the cytochemical reaction product is highlighted; c, the area of the nucleus is highlighted. Staining for cationic protein after M. Alfert and I. Geschwind [Butenko et al., 1974]

CFI is a convenient morphometric parameter: it is relative and allows measurements to be taken regardless of the smear density. At the same time, this index is not informative enough and requires an addition to the calculation formula. Apparently, it will be more accurate if the area of the nucleus (Fig. 2c) is subtracted from the cell area (Fig. 2a), and this difference is divided by the total area of the structures measured (stained CP granules) (Fig. 2b). In this case, it becomes possible to estimate the intensity of filling with the active substance directly in the volume of the cell cytoplasm, not in the entire cell volume. ICI shortcoming is that it requires the analysis of cells freely located in the visible field, so that the optical density of the stained substance does not change when cells overlap or are compressed.

The results of determining ACC, ICI, and CFI in granulocytes of the bottlenose dolphin are provided in Figs 3 and 4. The amount of CP-containing leukocytes (CP + leukocytes), intensity of staining, and degree of cell filling (CFI) with the cytochemical reaction product (ICI) change in these mammals with age. ACC values of bottlenose dolphins are higher than those of gray and harp seals, but lower than those of humans (Fig. 3).

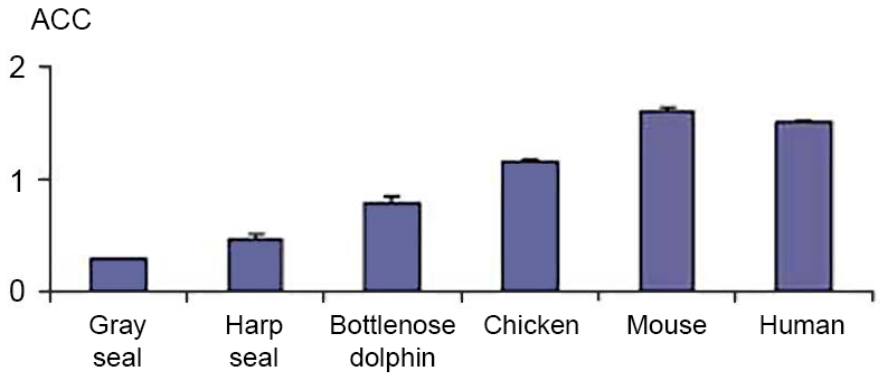

Fig. 3. The content of cationic protein in granulocytes of adult animals of different species (according to: [Budyka et al., 2009; Kletikova, 2010; Stoiko, Ermakov, 2004])

We introduced two more coefficients. Thus, mean values of CFI and ICI for samples of 200 granulocytes were expressed as the cell filling index coefficient (cCFI) and integral cytochemical index coefficient (cICI). These two parameters were used to compare groups and individual species of animals that differ significantly in protein activity in cells (ACC is applied for the same purpose). Specifically, in female bottlenose dolphins aged 4 to 5 years (circled in Fig. 4), against the backdrop of a high CFI value (*i. e.*, at the highest intensity of filling the cytoplasm of CP + leukocytes with stained granules) and a medium ICI value, the lowest ACC, cCFI, and cICI are observed which is due to a low percentage of CP + leukocytes in a mammal. Such individual fluctuations in the distribution of the active substance in leukocytes (by CFI) in bottlenose dolphins indicate an age-related decrease according to the trend line on the graph. Averaging the data obtained, both on the intensity of cell filling with granules of the active substance and on the degree of protein staining in cells, shows as follows: the amount of CP in granulocytes of an animal changes slightly with age. In seals, with medium ACC values, the content of active cells is high, but the intensity of filling with the active substance is low or medium.

The above-described indices and coefficients have their advantages and shortcomings. Application of ICI requires strict adherence to a staining technique in terms of the ratio of staining components and time of the procedure, as well as the use of non-overlapping cells. The core is that smear compaction leads to the stain thickening and, accordingly, to misinterpretation of the results obtained. CFI is convenient to use on any smears regardless of their density. Determination of all morphometric indicators is time-consuming, but computer technologies and automatic measurement programs allow to reduce time costs. Importantly, the use of ICI and CFI separately provides few data. It is way more reasonable to apply them combined with each other and with several other qualitative and quantitative parameters to assess the functional activity of the substance in studied cells. Consequently, comparative studies of the functional state of an animal organism based on various color-and-brightness characteristics of cells require calculations of additional indices and coefficients, as shown on the example of determining the content of CP in the bottlenose dolphin granulocytes. These indicators can be effectively used not only in assessing the content of CP in blood cells, but also in establishing the activity of other enzymes (alkaline phosphatase, succinate dehydrogenase, myeloperoxidase, *etc.*).

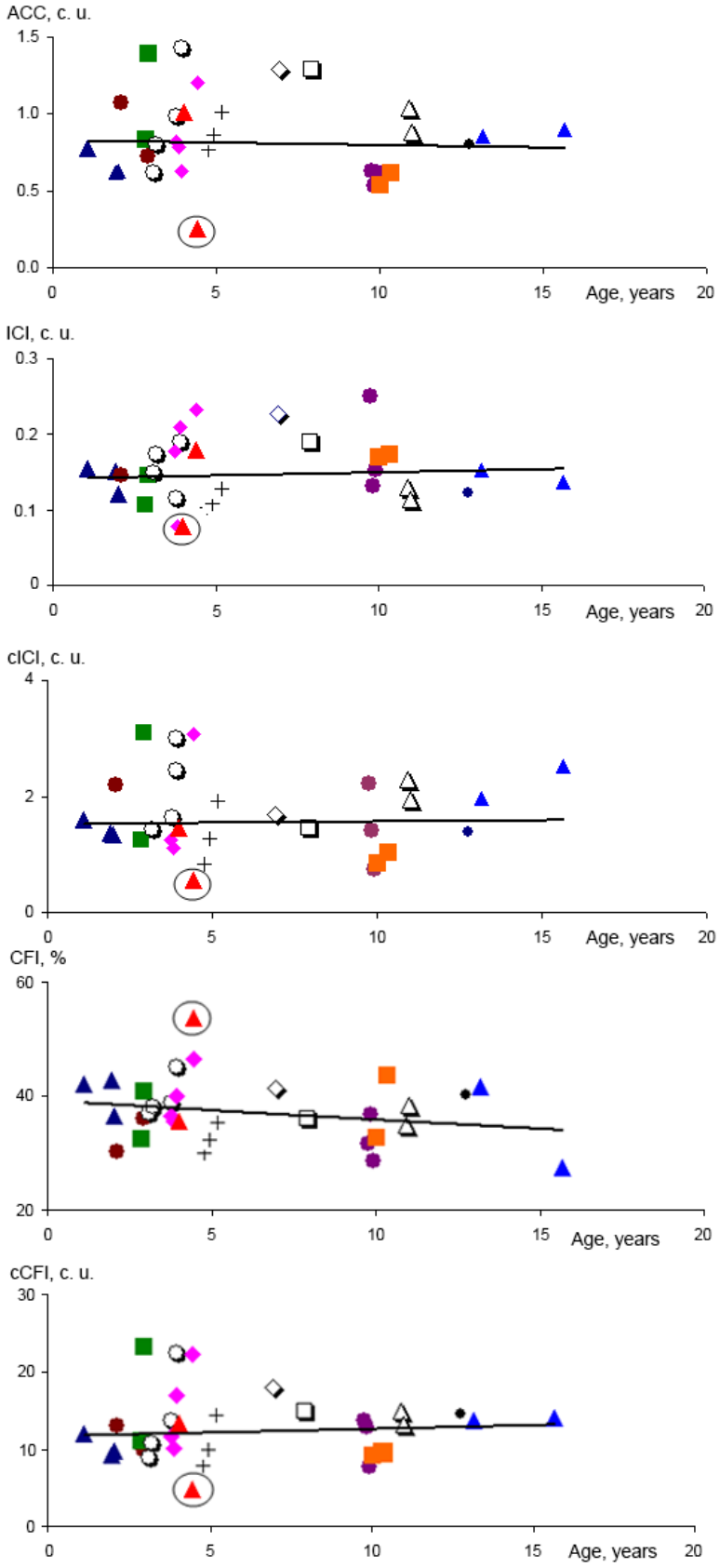

Fig. 4. Age-related changes in cytochemical parameters of the content of cationic protein in granulocytes of bottlenose dolphins (each individual animal is marked with its own symbol)

Morphometric measurement of a cell as a separate structure allows determining individual parameters for each organism. Despite the labor intensity of additional measurements and calculations when applying computer morphometry, indices provide accurate quantitative data on the content of substances in cells. The results of analysis of morphometric cellular parameters of marine mammals can be an important source of additional information for assessing the immunological status of animals. This is especially relevant for pinnipeds and cetaceans during their adaptation and long-term maintenance in oceanariums and dolphinariums. Biomaterial obtained from marine mammals in their natural habitat is often very disparate in age, sex, time, weight, and species characteristics of animals or even spoiled or insufficient in volume (only blood smears are preserved). Precision and maximum information content of microscopy of blood of animals allow carrying out comparative age, intraspecific, and interspecies studies of marine mammals.

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ИНДЕКСЫ В ОЦЕНКЕ ФУНКЦИОНАЛЬНОЙ АКТИВНОСТИ КЛЕТОК КРОВИ ДЕЛЬФИНА-АФАЛИНЫ *TURSIOPS TRUNCATUS* (MONTAGU, 1821)

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Содержание катионного белка в гранулоцитах дельфина-афалины *Tursiops truncatus* (Montagu, 1821) определяли методом расчёта среднего цитохимического коэффициента. Обоснованы его недостатки при визуальном установлении интенсивности окрашивания продукта цитохимической реакции на препаратах крови и при распределении клеток на группы по количеству в них белка. Применены современные методы оценки активности вещества в клетке с использованием компьютерных программ и светового микроскопа, что позволяет минимизировать погрешности морфометрических измерений объектов. Рассчитаны индивидуальные параметры по степени заполнения и интенсивности окрашивания катионного

белка в гранулоцитах у афалин с учётом и без учёта содержания белка во всём объёме крови. Такие показатели позволяют проводить сравнительные возрастные, внутри- и межвидовые исследования животных. Установлено, что содержание катионного белка в гранулоцитах может сильно различаться у разных особей афалин, а с возрастом его количество меняется незначительно.

Ключевые слова: морфометрия, средний цитохимический коэффициент, интегральный цитохимический показатель, катионный белок, гранулоциты, афалина