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Cuticle sclerotization precursor N-β-alanyldopamine also plays a role in the regulation of larval phototactic behaviour in *Drosophila melanogaster* (Diptera: Drosophilidae)

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El precursor de la esclerotización de la cutícula N-β-alanildopamina también participa en la regulación del comportamiento fototáctico de las larvas de *Drosophila melanogaster* (Diptera: Drosophilidae)

RESUMEN. La fototaxis es un comportamiento rígido y estereotipado en los insectos. La respuesta de las larvas a la luz se puede evaluar mediante métodos relativamente simples, rápidos y repetitivos. Mutantes de *Drosophila melanogaster* Meigen (Diptera: Drosophilidae) permiten estudiar la respuesta de las larvas a estímulos sensoriales. En las larvas la respuesta a la luz está controlada por mecanismos circadianos. Analizamos la respuesta a la luz empleando larvas mutantes de *D. melanogaster* para el metabolismo de los N-β-alanil derivados (mutantes *ebony* y *tan*). Los adultos *ebony* muestran déficits de comportamiento: ritmos circadianos anormales y fuertes defectos en la visión; el mutante *tan* muestra déficit leve en la visión. Nuestros resultados mostraron que las larvas *ebony* presentaron una respuesta aleatoria a la luz, mientras que las de *tan* mostraron una respuesta similar a las salvajes. Evaluamos si los N-β-alanil derivados están involucrados en esta respuesta, alimentándolas con N-β-alanildopamina (NBAD), N-β-alanilhistamina (carcinina) o dopamina (DA). NBAD restauró la respuesta normal a la luz en larvas *ebony*; carcinina y DA no produjeron efecto. Los resultados sugieren, por primera vez, que el NBAD estaría implicado en la respuesta de las larvas a la luz, actuando sobre el sistema visual o mediante el control circadiano de ésta.

PALABRAS CLAVE. Diptera**.** Fototaxia. N-β-alanildopamina. Mutante *ebony*.

ABSTRACT. Phototaxis is a well-studied insect behaviour described as rigid and stereotyped. Larval response to light can be assessed by a fast and highly repetitive assay like a half-dark/half-illuminated plate. *Drosophila melanogaster* Meigen (Diptera: Drosophilidae) mutants can be used to study the response of larvae to sensory stimuli. Adults of the *ebony* mutant show behavioural deficits such as abnormal circadian rhythms and severe defects in vision, whereas the *tan* mutants show a slight deficiency in vision. We analysed the response of *D. melanogaster* mutant larvae for N-β-alanyl derivatives metabolism (*ebony* and *tan*) to determine whether they also show abnormal phototactic behaviour. Our results showed that *ebony* larvae exhibited a random response to light, whereas *tan* larvae showed a similar response to wild-type larvae. To assess if N-β-alanyl derivatives metabolism might be involved in the response of larvae to light, we fed them with N-β-alanyldopamine (NBAD), N-β-alanylhistamine (carcinine) or dopamine. NBAD restored the wt response in *ebony* larvae, whereas carcinine and DA produced no effect. These results suggest, for the first

time, that NBAD might act as a neuroactive compound involved in the phototactic response of the *D. melanogaster* larvae.

KEYWORDS. Diptera. *ebony* mutant. N-β-alanyldopamine. Phototaxis.

INTRODUCTION

The larvae of the fruit fly *Drosophila melanogaster* Meigen (Diptera: Drosophilidae) spend most of their life feeding inside decaying fruits. They are strongly repelled by light and seek dark or less light-exposed surroundings (Humberg & Sprecher, 2017). The avoidance of light by fly larvae is a classic paradigm for assessing sensorimotor behaviour (Kane et al., 2013). First-instar to early 3rd instar larvae are photophobic and avoid light, whereas immediately before pupariation, they change to a photophilic behaviour, which they maintain throughout their adult stage (Sawin-McCormack et al., 1995; Mazzoni et al., 2005). Among the neurological and hormonal regulators of this behaviour, there are enzymes that synthesize neuroactive compounds that contribute to regulating the 24 h behavioural rhythms of the animals (Handler & Konopka, 1979; Silver et al., 1996). The enzyme N-β-alanyldopamine synthase (NBAD-synthase), also known as Ebony protein in *D. melanogaster*, is responsible for the synthesis of N-β-alanyldopamine (NBAD) (Wright, 1987; Wappner et al., 1996), the main tanning precursor of insect brown cuticles (Hopkins & Kramer, 1992). This enzyme also synthesizes other β-alanyl derivatives (Pérez et al., 2002, 2004; Richardt et al., 2003) and is involved in the circadian regulation of locomotor activity in adults (Suh & Jackson, 2007; Rossi et al., 2015). In diptera larvae, this enzymatic activity is present only in the nervous system (Richardt et al., 2002; Pérez et al., 2004), whereas it is absent in the integument until the beginning of pupariation (Pérez et al., 2004). In adult flies NBAD-synthase is expressed in the integument to sclerotize the cuticle only during the first hours following emergence, and then the enzymatic activity is no more detected. On the other hand, in the nervous system, its activity is present during the whole adult life synthesizing NBAD and carcinine (N-β-alanylhistamine). The other enzyme of this metabolism is NBAD-hydrolase (or Tan protein) which hydrolyses the N-β-alany derivatives (Borycz et al., 2002; Pérez et al., 2010), and its activity is present during the whole life cycle in the integument and in the nervous system. This enzyme has been less studied than NBAD-synthase (Pérez et al., 2011). In the nervous system, these two enzymes participate in a recycling pathway of neurotransmitters, mainly dopamine (DA), histamine (HA) (Borycz et al., 2003), and possibly also serotonin and octopamine (Pérez et al., 2004). The enzyme NBAD-synthase is found in glial cells and inactivates the action of DA and HA by conjugating them with β-alanine. In contrast, NBAD-hydrolase is present in neurons and restores the levels of DA and HA (Borycz et al., 2002; Perez et al., 2010, 2011). It has been shown that mutant flies for the NBAD metabolism have abnormal levels of neurotransmitters in the nervous system. *ebony* mutants, which lack NBAD-synthase activity, show higher levels of DA and reduced levels of NBAD (Fig. 1) (Hodgetts & Konopka, 1973; Walter et al., 1996; Gruntenko et al., 2004). The reciprocal mutant *tan*, which lacks NBAD-hydrolase, shows reduced levels of DA and increased levels of NBAD (Fig. 1) (Konopka, 1972; Walter et al., 1996; Pérez et al., 2021). In order to analyse whether the abnormal levels of neurotransmitters could affect mutant flies' response to light, we characterized *ebony1* and *tan1* larval photobehaviour. Moreover, due to the function of NBAD-synthase in the nervous system and its role in modulating circadian activity (Suh & Jackson, 2007; Rossi et al., 2015), we decided to explore whether its substrate DA or the products NBAD or carcinine might be involved in the phototactic response of the early 3rd instar larvae. We demonstrated that dopamine and carcinine produced no effect in the phototactic response of *ebony* larvae whereas larvae fed with NBAD behaved photophobic as wt larvae. It is well known the role of NBAD in cuticle sclerotization (Hopkins & Kramer, 1992), but its role in the nervous system is still elusive. Our study shed light on the metabolism of NBAD in the nervous system, where this compound is present (Krueger et al., 1990; Denno et al., 2016), but its function or role remains unknown.

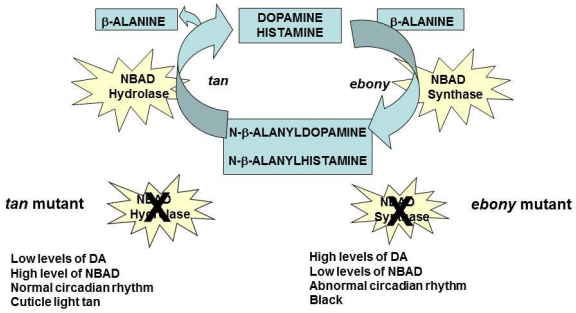

Fig. 1. N-β-alanyl metabolism and characteristics of *D. melanogaster* mutants *ebony* and *tan*. Schematic representation of the synthesis and hydrolysis of NBAD and carcinine. The positions of the enzymes NBAD-synthase and NBAD-hydrolase are indicated. The main physiological characteristics of both mutants are reported below.

MATERIAL AND METHODS

***Drosophila melanogaster* strains.** *D. melanogaster* wild-type Canton S (wt) and mutant strains *ebony1* (*e1*) and *tan1* (*t1*) were from Bloomington Stock Center. Larvae were kept at 23ºC and entrained in LD cycles as in Suh & Jackson (2007), since eclosion until the 3rd instar (12/12

light/dark cycle on a commercial fly medium 4.24 Instant Drosophila Medium - Carolina Biological Supply Co., USA). The larvae were kept at this temperature with the aim of slowing down larval development and thus being able to more easily collect synchronized individuals at the same age. In addition, *ebony1* mutant shows a more marked arhythmicity between 20-24 °C (Newby & Jackson, 1991). Larvae were transferred to constant darkness (DD) for 24 h and then tested for their ability to avoid light (Mazzoni et al., 2005; Chiu et al., 2010).

Chemicals. Dopamine (Cat Nº H-8502) and carcinine (Cat Nº C-2321) were purchased from Sigma Chemical Co; N-β-alanyldopamine was synthesized by Dr J.M. Aguirre (Luján University, Argentina) as described by Yamasaki et al. (1990).

Phototactic studies. Phototactic response studies were performed as described by Lilly & Carlson (1989) with slight modifications. Fourteen-centimetre diameter Petri dishes containing 100 ml of 2% agar were used to determine the phototactic responses. The dishes were divided into four zones, two black and two translucent (Fig. 2 a). Black zones were made by adding India ink (Pelikan, T-17, Argentina) to the agar and covering the outer face of the dish with aluminium foil to avoid light passing through. After the agar was solidified, translucent agar was poured onto the surface to avoid the presence of a potential chemosensory stimulus in the dye. After the selection of optimal light intensity, the experiments were carried out at 400 lux, measured with a LI-COR 250A lux meter (LI-COR Inc, USA). Twenty early 3rd instar larvae were used in every independent experiment. Third instar larvae were collected and rinsed with 0.1 M PBS buffer pH 7.3; they were placed in the middle of the dish and let to explore the surface for five minutes. Fresh agar was used for each experiment. The experiments were carried out between *Zeitgeber* time (ZT) ZT-3 and ZT-5 (11:00-13:00 h) in a dark room and the only light available was the one below the dishes.

Response index. The response index (RI) in the phototaxis test was calculated as reported by Gong (2009) and Borycz et al. (2018); PI = (number of larvae in the light half - number of larvae in the dark half) / (number of larvae in the dark half + number of larvae in the light half), where RI = -1 represents a highly photophobic behaviour, whereas RI = 1 describes a positive phototactic phenotype.

Dopamine and N-β-alanyl derivatives larvae feeding. Larvae food was mixed with blue food dye and supplemented with 2.5 mM dopamine, carcinine, and NBAD dissolved in 500 mM acetic acid. Larvae were fed 2 h before the assay of the phototactic response. Control was performed by feeding the larvae only with the vehicle (acetic acid) to discard any effect caused by this acid. As larvae are translucent we were able to verify whether they have ingested the food (containing blue dye and any drug) by observing under a binocular microscope if their digestive tracts were dyed blue. We used in the assays the larvae with blue-dyed digestive tracts. Larvae without blue-dyed digestive tracts were discarded.

Statistical analysis. Results are presented as barplots showing the mean value plus the minimum to the maximum standard error of the mean (SEM); n indicates the number of independent replicates. Response index results are presented as boxplots. The non-parametric Kruskal-Wallis and Dunn's Tests were used to compare treatments to their corresponding control (wt). For multiple comparisons of treatment effects between strains, t-test was applied. The Benjamini, Krieger and Yekutieli False Discovery Method (FDR) was applied to correct for multiple comparisons, using the desired FDR (Q) of 5%. Adjusted p-value differences of <0.05 were considered statistically significant ($p > 0.05$ (ns), $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***), $p \leq 0.0001$ (****). GraphPad Prism statistical software was used (version 8.2.1).

RESULTS

The phototactic responses of *D. melanogaster* wt, *ebony1* and *tan1* mutant larvae were analysed at 400 lux. Mazzoni et al. (2005) demonstrated that *D. melanogaster* wt larvae showed normal sensitivity to light at intermediate intensity (>150 and <600 lux), whereas at low light intensities (less than 150 lux), they can barely distinguish between light and dark thus they almost not differentiate between the light and dark sides of the plate. Similarly, the photophobicity of the wt larvae also diminishes at high light intensities (more than 600 lux). According to these results, we carried out the assays at 400 lux. Wt and *tan1* larvae showed similar phototactic behaviour: 91% and 85% of the individuals, respectively, moved to the dark zones, thus indicating that *tan1* larvae are strongly photophobic like wt larvae; the response index for wt larvae was -0.828 +/- 0.020, whereas for *tan1* was -0.710 +/- 0.083. In contrast, *ebony1* larvae seemed to show a random distribution since only 53% of larvae moved to the dark zones during the five min of the experiment, and the remaining 47% moved to the illuminated zones; the response index of these mutant larvae was -0.077 +/- 0.051 (Fig. 2 b).

In order to assess whether the abnormal response of *ebony1* larvae was due to a misbalance of DA, carcinine or NBAD, we analysed the phototactic response of *ebony1* larvae fed two hours before the test with fly medium containing one of these substances dissolved in acetic acid. According with Mazzoni et al. (2005) the sensitivity of wild-type larvae to light follows a circadian rhythm. Thus, to carry out the assay, larvae were entrained in LD cycles since eclosion until one day before the test and then shifted into DD, to evaluate their ability to avoid light. Larvae were entrained in LD cycle because more relevant information could be obtained with this protocol instead of entraining the larvae in constant conditions, such as DD (Eelderink-Chen et al., 2015). We

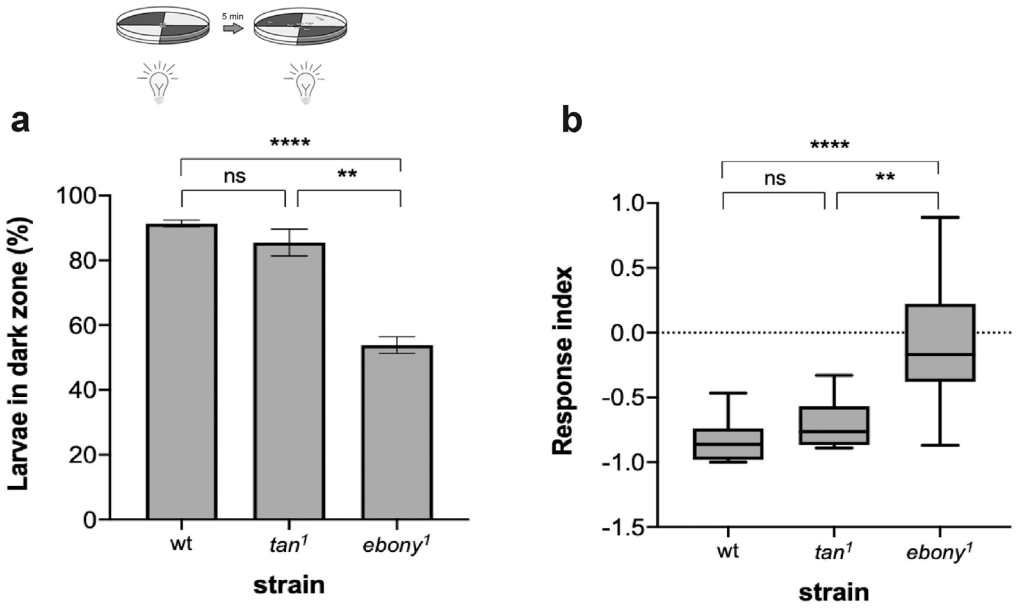

Fig. 2. Mutants phototactic response. a. Upper panel: schematic representation of the phototactic study: the plates were situated in a dark room illuminated only by a light coming from below. Lower panel: the graph shows the mean percentage of larvae in the dark zone for wt, *tan1* and *ebony1* strains (n ≥ 6 independent experiments, Kruskal–Wallis and Dunn's multiple comparison tests against wt strain (*tan1* p = 0.6919 and *ebony1* p < 0.0001); comparison tests *tan1* against *ebony1* strain p=0.0091). b. Response index of the 3 strains; the upper and lower boxes indicate the first and third quartiles, the thick line is the median. The upper and lower whiskers indicate 1.59 the interquartile range (*tan1* p >0.9999 and *ebony1* p < 0.0001: comparison tests *tan1* against *ebony1* strain: p=0.0096).

decided to follow the most commonly used entrainment condition (12:12 LD) to carry out tests regarding circadian studies (Chiu et al., 2010). Fig. 3 shows that wt larvae behaviour was not affected by the addition of DA, carcinine or NBAD, as they maintained a photophobic response in every condition, with more than 80% of the larvae situated on the dark areas. Similarly, *ebony1* seemed not to respond to the treatment with DA or carcinine, as they showed a random response, similar to the non-treated *ebony1* larvae. Surprisingly, *ebony1* larvae that previously were fed with NBAD moved mainly to the dark zone, thus behaving negatively phototactic as wt larvae. Therefore, after the treatment with NBAD no mutant behavioural phenotype was observed in *ebony* larvae. Phototactic indexes show that only *ebony1* larvae fed with NBAD have changed the response to light, turning from a random to a photophobic response (Fig. 2). As *tan* larvae showed similar phototactic response as wt larvae we did not test whether DA, carcinine or NBAD modify their phototactic response. Besides, as previously mentioned, this mutant shows high levels of NBAD; feeding it with this compound should produce no effect. Our aim was to assess if any of the molecules involved in the N-β-alanyl derivates metabolism was able to restore the normal phototactic response in mutant larvae.

DISCUSION

In the present work, we studied the phototactic behaviour of *D. melanogaster* larvae mutant for the N-β-alanyl derivatives metabolism (Fig. 2) and the possible role of these derivatives in the phototaxis of larvae (Fig. 3). Our results strongly indicate that the abnormal phototaxis of *ebony1* larvae is due to a physiological impairment related to NBAD deficiency. The fact that the addition of carcinine, which is an N-β-alanyl derivative involved in visual physiology in adults, did not change the response of these larvae indicates that the response to NBAD is specific (Fig. 3). DA (precursor of NBAD synthesis) does not seem to affect the phototactic response of the larvae, as treatment with this compound did not affect larvae behaviour (Fig. 3). This last result is in agreement with previous results that showed that flies with low DA levels like *tan1,* or flies treated with tyrosine hydroxylase inhibitors (Neckameyer, 1996) showed normal responses to light, thus indicating that DA might not affect phototactic behaviour. On the other hand, the effects of NBAD suggest that the abnormal response of *ebony1* is due to a behavioural defect and that it may be related to the lack of this beta-alanyl derivative. Glia cells containing Ebony enzymatic activity are localized close to clock cell projections, where the regulatory clock genes *per* and *tim* are expressed. Moreover, Ebony enzymatic activity is required within the glia for the clock control of locomotor activity (Suh & Jackson, 2007). According to the results of these authors, Ebony acts downstream of the clock to control locomotor activity and glial cells expressing Ebony are positioned near DA and serotonin neurons of the larval and adult brains, which strongly suggests that these glial cells are required for the modulation of aminergic functions. Interestingly *per* and *tim* mutant larvae show reduced light avoidance, as it

occurs with *ebony1* mutants, highlighting the notion that the circadian clock regulates this response (Mazzoni et al., 2005; Gong, 2009). The fact that *tan1*, which presents increased levels of NBAD, showed a normal response to light (Fig. 2), also reinforces the idea that NBAD contributes to modulating or regulating the larval phototactic behaviour, independently of the available DA in neurons.

Using a reliable and repetitive behavioural assay we showed that *D. melanogaster ebony1* larvae, mutant for NBAD metabolism have an abnormal phototactic behaviour. We also demonstrated that feeding the larvae with NBAD resulted in the recovery of phototactic response. As far as we know there is no evidence in the literature that any N-β-alanyl derivative might have bioactivity in nervous system, but our results show, for the first time, evidence of the involvement of NBAD in the regulation of phototaxis in *Drosophila* larvae. The link between NBAD signalling and the circadian clock (Suh & Jackson, 2007) suggests a homeostatic mechanism for the regulation of photosensitivity in *Drosophila* larvae.

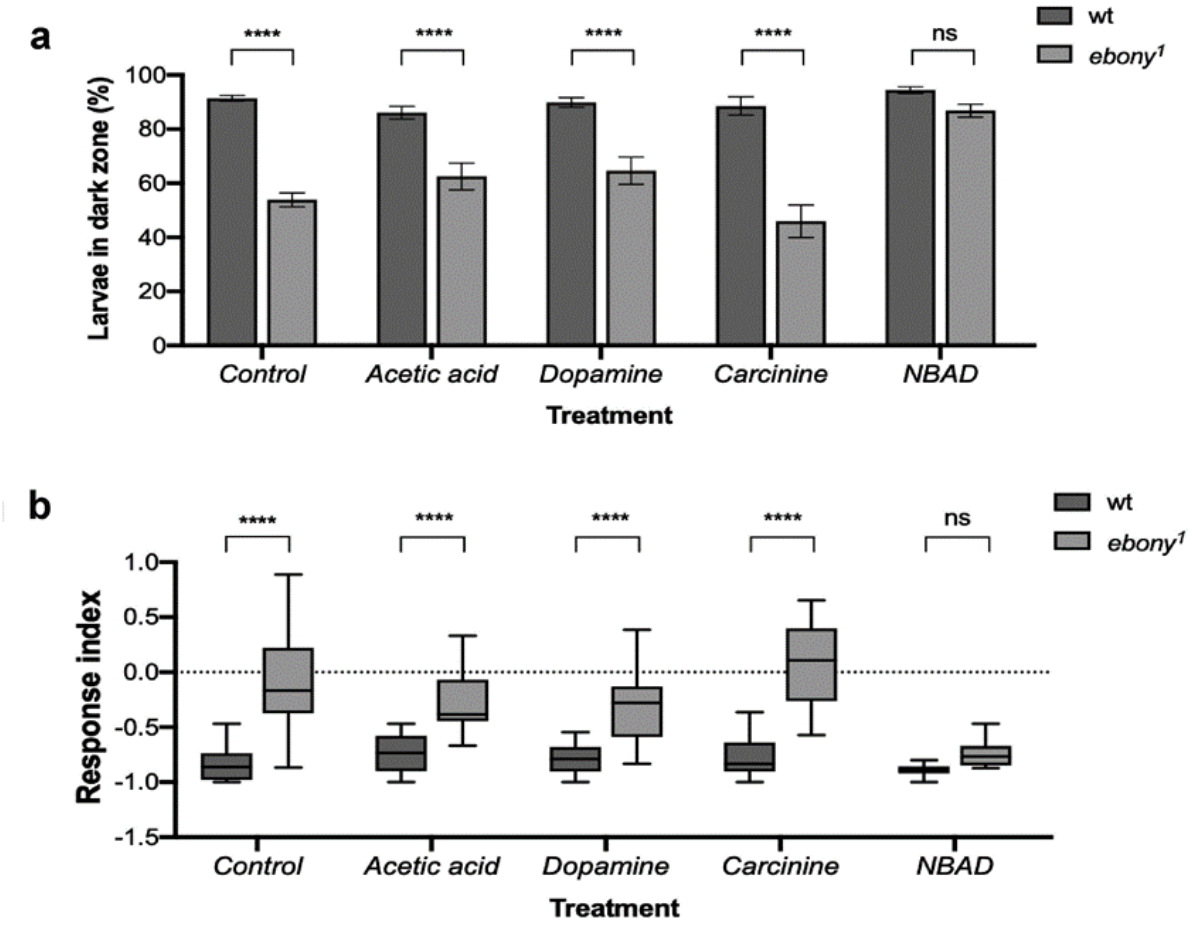

Fig. 3. Effect of DA, NBAD and carcinine on the phototactic response of wt and *ebony1*. a. The graph shows the mean percentage of larvae in the dark zone for wt and *ebony1* strains that were fed two hours before the assays with different compounds (n ≥ 8 independent experiments, multiple t-tests with FDR of 5%. Control: $p < 0.000001$, Acetic acid: $p= 0.000017$, Dopamine: $p= 0.000003$, Carcinine: $p< 0.000001$, NBAD: $p= 0.065713$). b. Response index: The upper and lower boxes indicate the first and third quartiles, the thick line is the median. The upper and lower whiskers indicate 1.59 the interquartile range (Control: $p < 0.000001$, Acetic acid: $p= 0.000019$, Dopamine: $p= 0.000003$, Carcinine: $p< 0.000001$, NBAD: $p= 0.065581$).

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