1   *Caenorhabditis elegans* **as an emerging model for studying the basic biology of**

2   **anorectic effects of nicotine**

3   Robert Sobkowiak[a]*, Piotr Kaczmarek[b], Mateusz Kowalski[b], Rafał Kabaciński[b], Andrzej

4   Lesicki[a]

5

6   [a] Department of Cell Biology, Adam Mickiewicz University, Faculty of Biology

7   ul. Umultowska 89, 61-614 Poznań, Poland

8   [b] Poznan University of Technology, Faculty of Electrical Engineering, Institute of Control and

9   Information Engineering, ul. Piotrowo 3a, 60-965 Poznań, Poland

10

11   * Corresponding author; tel.: +48 61 8295822; fax: +48 61 8295829;

12   e-mail: robsob@amu.edu.pl

13

14

17

18   **Competing interests**: The authors have declared that no competing interests exist.

19

20   **Abstract**

21   Nicotine decreases food intake, and smokers often report that they smoke to control

22   their weight. To see whether similar phenomena could be observed in the model organism

23   *Caenorhabditis elegans*, we challenged drug-naïve nematodes with a chronic low (0.01 mM)

24   and high (1 mM) nicotine concentration for 55 h (from hatching to adulthood). After that, we

25   recorded changes in their behavior in a nicotine gradient, where they could choose a desired

26   nicotine concentration. By using a combination of behavioral and morphometric methods, we

27   found that both nicotine and food modulate worm behavior. In the presence of food the

28   nematodes adapted to the low nicotine concentration, when placed in the gradient, chose a

29   similar nicotine concentration like *C. elegans* adapted to the high nicotine concentration.

30   However, in the absence of food, the nematodes adapted to the low nicotine concentration,

31   when placed in the gradient of this alkaloid, chose a similar nicotine concentration like naïve

32   worms. The nematodes growing up in the presence of high concentrations of nicotine had a

33   statistically smaller body size, compared to the control condition, and the presence of food did

34   not cause any enhanced slowing movement. These results provide a platform for more

35   detailed molecular and cellular studies of nicotine addiction and food intake in this model

36   organism.

37

38

41

1   Introduction

Tobacco smoking is the largest single preventable cause of many chronic diseases and death (WHO, 2012). Most of the tobacco cigarette's toxicity is related to the combustion process. Although tobacco smoking has declined since the 1950s, the use of e-cigarettes has increased, attracting both former tobacco smokers and never smokers. Regardless of nicotine source, method for delivering, and other concomitant substances, nicotine is the most biologically active ingredient of seriously harmful tobacco smoke and the potentially harmful e-cigarette aerosol (Grana et al., 2014; Schuller, 2009; Sobkowiak and Lesicki, 2011).

Some smokers report that they smoke as a method of weight control (Nichter et al., 2004). Indeed, smokers have a notably lower body mass index than nonsmokers and gain weight when they quit (Filozof et al., 2004; Jo et al., 2002; Mineur et al., 2011). These effects on body weight have been attributed to nicotine in tobacco, because nicotine decreases food intake in animal models. In humans, nicotine has some effects on peripheral energy metabolism (Filozof et al., 2004; Jo et al., 2002) and this was also observed in the model nematode *Caenorhabditis elegans* (Sobkowiak et al., 2016).

Nicotine competes with acetylcholine for binding to specific membrane receptors, so-called nicotinic cholinergic receptors (nAChRs). They are widely expressed in the nervous system and skeletal muscle. Nicotinic receptors are also present in many cell types, e.g. epithelial, blood, fat, and cancer cells (Liu et al., 2004; Sobkowiak and Lesicki, 2011). Also nicotine metabolites have significant effects on the body (Benowitz et al., 2009; Sobkowiak and Lesicki, 2013). Early nicotine exposure has been associated with many long-term consequences that include neuroanatomical alterations as well as behavioral and cognitive deficits (Rose et al., 2013). Moreover, nicotine is a highly addictive substance with negative effects on animal and human brain development, which is still ongoing in adolescence (Benowitz, 2010; Grana et al., 2014).

Locomotion reflects the integration of many aspects of both the environment and the internal state of the worm, and therefore can be a sensitive measure of behavioral state after nicotine application. As an animal travels through its environment, its nervous system detects sensory cues, evaluates them based on context and the experience of the animal, and converts the information into adaptive movement. *C. elegans* has behavioral states that are evident as long-term locomotor patterns that differ in well-fed or starved animals. When feeding on a bacterial lawn, *C. elegans* switches between 2 behavioral patterns. It spends 80% of its time dwelling, i.e. feeding on bacteria while moving slowly and staying in a restricted area. At rare intervals, however, it switches into an alternative behavioral state called roaming, which

3

76   involves rapid locomotion across the lawn. When removed from the bacterial lawn, *C.*

77   *elegans* switches to a behavioral state in which it moves rapidly and reverses frequently. This

78   strategy is called pivoting, area-restricted search, or local search, and has the same basic run-

79   and-pirouette components as directed chemotaxis. Over the next 30 min, reversals are

80   suppressed, shifting the animals to another behavioral state, called traveling or dispersal

81   (Bargmann, 2006 and the papers cited therein).

82   Worms navigate to favorable conditions by chemotaxis and aerotaxis (Gray et al.,

83   2005). *C. elegans* shows chemotaxis to various odorants and water-soluble chemoattractants,

84   like nicotine, by sensing them mainly with sensory neurons whose sensory endings are

85   located in amphid sensory organs at the anterior tip of the animal. The strategy of chemotaxis

86   in this organism was previously studied by Pierce-Shimomura et al. (1999), who found the

87   "pirouette strategy" for chemotaxis to water-soluble chemoattractants. Iino and Yoshida

88   (2009) reported the discovery of a second mechanism for chemotaxis, called the weathervane

89   mechanism. In that strategy, the animals respond to a spatial gradient of the chemoattractant

90   and gradually curve toward a higher concentration of the chemical.

91   *Caenorhabditis elegans* is a powerful genetic model for studying various questions in

92   neurobiology, including nicotine dependence. For example, it has been reported that *C.*

93   *elegans* responds to nicotine in both a concentration-dependent and a time-dependent manner,

94   and shows a wide range of behavioral responses to nicotine (Feng et al., 2006; Sobkowiak et

95   al., 2011). These include a continuous higher locomotion speed (acute response), tolerance,

96   withdrawal, and sensitization via nAChR (Feng et al., 2006). Dual effects of nicotine on

97   locomotion speed, which are dependent on differences in its dosage and treatment duration,

98   have also been revealed (Sobkowiak et al., 2011). Moreover, *C. elegans* is capable of

99   navigating up a nicotine gradient (Sellings et al., 2013).

100   In the current study we observed spontaneous behavior in the presence or absence of

101   food as well as a nicotine gradient to describe the effects of low and high nicotine

102   concentration exposure during larval development in adult *C. elegans*.

103

2   Results

2.1   Body size of worms

Nicotine affected the body size of nematodes. Worms challenged from hatching to adulthood (55 h, 22°C) with 1 mM nicotine were smaller than in control conditions (Fig. 3). The worms adapted to the high nicotine concentration (1 mM) were about 5% shorter (Fig. 3A), with about 12% smaller surface (Fig. 3B), and about 22% smaller body volume, as compared to control nematodes (Fig. 3C). The nematodes adapted to a low nicotine concentration (0.01 mM) were on average about 4.5% longer (Fig. 3a), with about 5.5% smaller volume (Fig. 3C), as compared to control nematodes. However, there was no statistically significant difference between these 2 groups.

2.2   Average values of factors describing worm behavior

2.2.1   Average speed of movement

The average speed of movement of well-fed *C. elegans* worms (Fig. 4A) was the fastest in the absence of food (F-nA0), and their locomotion slowed down when food was present (F+). A similar effect of slowing the movement, but also in the absence of food, was evoked by the gradient of nicotine (F-N).

In the presence of food on the nicotine-free Petri dish, there was no statistically significant difference in locomotion speed between the nematodes having the first contact with this alkaloid (naïve worms) and nematodes adapted to 0.01 mM nicotine (Fig. 4A, F+nA0 vs. F+nA0.01). In the presence of food and without nicotine in the center of the assay plate, the worms adapted to 0 mM nicotine (F+nA0) and those adapted to 0.01 mM nicotine (F+nA0.01) moved with the same speed (no significant differences, Fig. 4A).

The worms adapted to 1 mM nicotine in the presence of nicotine gradient moved with the same speed independently of the presence or absence of food (Fig. 4A, no significant differences between F+NA1 and F-NA1). It is noteworthy that the nematodes adapted to 1 mM nicotine (F+nA1) in the presence of food unexpectedly moved farthest from the start position and the presence of food appeared to stimulate the process.

5

2.2.2 Average distance from the start position

Nematodes moved away from the starting point in a range of 3 mm (F+KA0) to 18 mm (F-NA0) (Fig. 4B). The presence of food in the absence of nicotine gradient evoked in naïve worms a tendency to keep close to the start position (Fig. 4B, F-nA0 vs. F+nA0). Median value of the distance from the starting point for the worms adapted to 0.01 mM nicotine was almost the same but the range of 25%-75% narrowed (Fig. 4B, F-nA0.01 vs. F+nA0.01).

In the presence of food we observed a linear tendency with a significantly ($p < 0.05$, Kruskal-Wallis ANOVA by ranks test) increased distance of worms from the starting point (Fig. 4B, F+nA0 lowest value, F+nA0.01, F+nA1 highest values).

We observed an opposite tendency when a gradient of nicotine appeared on the plate. Independently of the presence or absence of food, nicotine extorted a decrease in the distance from the start position in line with the increase in nicotine concentration to which the worms were earlier adapted (Fig. 4B, F+NA0, F+NA0.01, F+NA1). Interestingly, we revealed no statistical difference in average distance from the start position between the worms adapted to 1 mM nicotine in the presence of food and absence of food (Fig. 4B, F+NA1 vs. F-NA1)

2.2.3 Average distance from the peak

The peak was the central point where nicotine or water was applied on the gradient assay plate. The radius in which nematodes were present, measured from the peak to worm location, was in the range from about 4 mm (F-NA0, F-NA0.01) to about 21 mm (F+nA0.01) (median value, Fig. 4C). When there was no nicotine in the center of the gradient assay plate, the shortest distance from the peak was observed for the worms adapted to 1 mM nicotine (F+nA1). When the gradient of nicotine appeared and in the absence of food, the worms adapted to 0 mM and 0.01 mM reached the peak and behaved in the same way (Fig. 4C F-NA0, F-NA0.01, no statistical difference). The worms adapted to 0.01 mM nicotine in the presence of food were not attracted to the peak and they chose food and kept distance like on the plates without nicotine (Fig. 4C, compare median values for F+nA0.01 vs. F+NA0.01 vs. F-NA0.01).

2.2.4 Average nicotine concentration preferred by worms

In our experiment, nematodes could select the concentration of nicotine in which they wanted to stay. Their movements depended on the presence of food and on adaptation to

161 nicotine. In the absence of food, worms preferred on gradient assay plates higher
162 concentrations of nicotine in comparison to the worms placed on plates without food (Fig.
163 4D). The nematodes adapted to the low concentration of nicotine (0.01 mM) behaved
164 differently in comparison to the nematodes adapted to the high concentration of nicotine (1
165 mM). The worms adapted to 1 mM chose the lowest nicotine concentration independently of
166 the presence or absence of food (Fig. 4D, F+NA1 and F-NA1). In the presence of food the
167 worms adapted to 0.01 mM nicotine behaved like the worms adapted to 1 mM nicotine (Fig.
168 4D, F+A0.01 vs. F+NA1, no statistical difference). However, in the absence of food, the
169 worms adapted to 0.01 mM nicotine behaved like the worms adapted to 0 mM nicotine (Fig.
170 4D, F-NA0 vs. F-A0.01, no statistical difference) and chose a very high concentration of
171 nicotine.

172 2.3 Time-dependent response of *C. elegans* to the nicotine gradient

173 2.3.1 Time-dependent speed of movement

174 The speed of the worms varied with time (Fig. 5). In most cases, we observed a
175 decreasing trend in the speed of their movements, and its frequent oscillations. In control
176 conditions, i.e. in the absence of a gradient of nicotine and food (F-nA0), we observed
177 oscillations of the speed, while the nematodes in variant F-nA0.01 show a downward trend in
178 the speed of movement (Fig. 5E). The long period of reduced locomotion activity was
179 observed particularly in control nematodes (Fig.Fig. 5b, time 500-1100 s) and those adapted
180 to 0.01 mM nicotine (Fig. 5E, time 2500-3600 s; Fig. 5G, time 700-1400 s).

181 2.3.2 Time-dependent changes in distance from the start position

182 Fig. 6 shows the variation in the distance from the start position of worms (measured
183 from the start position to the actual worm location). Generally during the experiment the
184 nematodes moved away from the starting point. In some experimental conditions, nematodes
185 remained at a relatively stable distance from the start position (Fig. 6B,F,K). This applied
186 especially to the worms that never had contact with nicotine before, and were transferred to a
187 Petri dish with bacteria and no nicotine gradient (Fig. 6C, F+nA0). Those worms tended to
188 move away from the starting point about 1200 s after application (.Fig. 6C). Farthest mean
189 distance from the start position was recorded for the nematodes adapted to 0.01 mM nicotine,
190 on the plate without nicotine and bacteria (variant F-nA0.01, Fig. 6E).

7

2.3.3   Time-dependent changes in distance from the peak

The distance between worms and the peak varied with time (Fig. 7), but was exceptionally constant for the nematodes adapted to 1 mM nicotine and placed on Petri dishes with food and nicotine gradient (F+NA1, Fig. 7I). Interesting behavior of nematodes was observed in variants F-NA0 and F-NA0.01 .Fig. 7B,F), where almost immediately after application they moved towards the central peak of nicotine.

2.3.4   Time-dependent changes in preferred concentration of nicotine

In a radial gradient of nicotine, control wild-type nematodes and those adapted to 0.01 mM nicotine usually reached the gradient peak by moving almost directly up the gradient (Fig. 8A,C, F-NA0 and F-NA0.01). The preference for higher concentrations appeared about 300 s after the application of nicotine in the center of the Petri plate. However, the nematodes adapted to 0.01 mM nicotine, when placed on plates with food, avoided nicotine for the first 900 s of experiment (Fig. 8D, time 0-1000s). Nematodes adapted to 1mM nicotine avoided nicotine for the first 1500 s of the experiment (Fig. 8E, f, time 0-1000s).

3   Discussion

206

207   In the experiments we used 3 groups of nematodes: naïve worms (which never had

208   contact with nicotine before), worms adapted to a low uniform nicotine concentration (0.01

209   mM) and to a high uniform nicotine concentration (1 mM) (Fig. 1B). The adaptation to

210   nicotine lasted from hatching to adulthood, and next we investigated the behavior of these

211   nematodes in a nicotine gradient (Fig. 1C). Nicotine was administered in the center of the

212   Petri plate in one bolus dose. Theoretically, the bolus of 1 µL of 580 mM nicotine diluted in

213   5.8 mL of medium in Petri dish diffuses to final 0.1 mM nicotine concentration and

214   disappearing of nicotine gradient after about 12 h. However, we started our experiments

215   immediately after administration of nicotine onto the Petri dish. In our previous study, in

216   uniform nicotine concentration, within the first 3600 s we observed the largest difference in

217   the behavior of control naïve nematodes and those in the presence of 0.1 mM nicotine

218   (Sobkowiak et al., 2011). In this study, we used a 10-fold lower (0.01 mM) and 10-fold higher

219   nicotine concentration (1 mM) during adaptation of worms. These 2 concentrations were

220   considered ''effective dosages'', as our previous report revealed that dosages in this range

221   cause changes in locomotion (Sobkowiak et al., 2011, see Fig. 2 in that article).

222   We performed chemotaxis assays by tracking individual worms in the radial Gaussian-

223   shaped gradients of nicotine. *C. elegans* has a highly developed chemosensory system that

224   enables it to detect a wide variety of volatile (olfactory) and water-soluble (gustatory) cues

225   associated with food and chemicals (Bargmann, 2006). During the experiments, the

226   nematodes were allowed to move freely around the Petri dishes, and able to select the desired

227   nicotine concentration. Sellings et al. (2013) suggest that nicotine acts as a rewarding

228   substance in *C. elegans*. The nematodes approach a point source of nicotine in a time-

229   dependent and concentration-dependent manner. Those authors revealed that wild-type worms

230   climb the nicotine gradient, and suggested that worms exposed to 50 mM nicotine can

231   approach appetitive stimuli, once removed from the nicotine source. This is in line with our

232   result, with the exception of the high nicotine concentration experimental variant. The naïve

233   animals and worms adapted to low nicotine concentration reached the peak of the gradient,

234   defined as a circular region with a radius of 5 mm located at the center of the plate (Fig. 2A,

235   Fig. 7B, F), and were trapped in the nicotine area when the nicotine concentration exceeded

236   500 mM at the beginning of experiment (Fig. 8A,B,C). The cuticle of *C. elegans* is a

237   significant barrier for drug permeability, thus the internal concentrations in the worm's body

238   fluid is likely to be substantially lower than the dosing medium concentration (Wolf and

239   Heberlein, 2003). However, high concentrations of nicotine induce muscle hypercontraction

9

240   paralysis (Matsuura et al., 2013; Sobkowiak et al., 2011) and cause rapid transient paralysis of

241   body wall muscles, manifested in an extremely low speed of locomotion (Fig. 5A, time 300-

242   900s; Fig. 5F, 1200-1600s). This suggests that the nematodes were affected by nicotine

243   toxicity and paralysis.

244   The naïve animals and those adapted to the low nicotine concentration tested in the

245   nicotine gradient were significantly more unlikely to reach the center when on the Petri dish

246   the food was in a uniform concentration (Fig. 5C and 8D,H). There were no statistically

247   significant differences in distance from the peak between the naïve animals and the worms

248   adapted to the low nicotine concentration (Fig. 4C, F-NA0 vs. F-NA0.01).

249   Due to the nature of the behavior of the nematode *C. elegans*, the distribution of all the

250   measured values was not normal. Therefore, for this type of distribution, an appropriate

251   measure of central tendency is the median. Nonparametric tests more easily demonstrate the

252   existence of a significant statistical difference, especially when a large amount of data is

253   available. In our study, a single experiment provides 7200 data, because records were taken

254   every 0.5 s during the 3600 s of the experiment. Additionally, the experiments were repeated

255   5 times, to increase the pool of results. Thus most of the experimental variants differed

256   significantly from one other. In our view, more biologically important are the results which do

257   not differ in a statistically significant way, despite the distinctly different experimental

258   conditions.

259   *Caenorhabditis elegans* chemotaxes to bacteria, its natural food source, by following

260   both water-soluble and volatile cues. Because of this difference in sensitivity, and because the

261   diffusion of small molecules through air is much more rapid than through water, it is likely

262   that volatile odors are used first for long-range chemotaxis, and later water-soluble attractants

263   are used for short-range chemotaxis (Bargmann, 2006). Many studies have shown that the

264   absence or presence of food markedly influences the average speed of wild-type worms (de

265   Bono and Bargmann, 1998; Ramot et al., 2008). Sawin et al. (2000) reported that the feeding

266   status (well-fed or starved) as well as the presence or absence of food (bacteria) affects the

267   rate of locomotion of *C. elegans*. Sawin et al. (2000) reported a decrease in the locomotory

268   rates of well-fed adult *C. elegans* on a bacterial lawn (food) compared with those on plates

269   lacking bacteria, and defined this response as the "basal slowing response". Nicotine exposure

270   alters behaviors in *C. elegans*, including pharyngeal pumping, which disturbs nutrition (Matta

271   et al., 2007). Pharynges that have been dissected from wild-type worms hypercontract in 0.1

272   mM nicotine (McKay et al., 2004). Nematodes in the presence of high concentrations of

273   nicotine have smaller body size (Fig. 3) probably because they eat less, as compared to

274   control nematodes, thereby releasing less serotonin, and do not show enhanced slowing on
275   food (Fig. 5A and 6K,L). The neurosecretory-motor neurons that have sensory endings in the
276   lumen of the pharynx (which thus might sense food) also synthesized serotonin (Chase and
277   Koelle, 2007). Serotonin is required for the so-called "enhanced slowing response" (Sawin et
278   al., 2000). *C. elegans* individuals adapted to the high nicotine concentration were smaller than
279   those in the control, probably because in the presence of food their serotonergic neurons did
280   not release an increased amount of serotonin, which did not inhibit the motor circuit to a
281   greater extent than in the basal slowing response to effect the enhanced slowing response (Fig.
282   4A F+nA0 vs. F+nA1). Without such serotonin release, the enhanced slowing response could
283   not occur. Thus on the plate with food, the worms adapted to the high nicotine concentration
284   moved faster than the control worms. The animals slow their locomotion rate dramatically
285   when they encounter a bacterial lawn (Fig. 5A and 6C,D,G,H) and, as mentioned above, the
286   enhanced slowing response requires serotonin (Sawin et al., 2000). Serotonin signaling thus
287   provides a mechanism to ensure that animals absolutely do not leave a food source once they
288   have encountered it (Chase and Koelle, 2007). That is probably why we observed a very short
289   distance from the start position for the control worms and a little larger distance for those
290   adapted to the low concentration to nicotine (Fig. 6C,G).

291   Sellings et al. (2013) were the first to reveal that nicotine serves as a primary motivating
292   stimulus in *C. elegans*. Their research suggests that dopamine (DA) transmission is important
293   in mediating the nicotine-motivated behaviors (Sellings et al., 2013). The presence of bacteria
294   is perceived by mechanosensory stimulation via 8 dopaminergic sensory neurons (Sawin et
295   al., 2000). The level of dopamine is higher in the presence of nicotine. We hypothesized that
296   in nematodes adapted to high nicotine concentration (1 mM), the alkaloid mimics bacterial
297   mechanosensory stimulation via sensors of dopaminergic neurons, thus nematodes eat less
298   and have smaller body size (Fig. 3A, F-nA0 vs. F-nA1). The dopamine signaling in well-fed
299   animals encourages the animal to stay in the proximity of food but still may permit limited
300   exploration for new or better food sources (Chase and Koelle, 2007). This is a likely
301   explanation why the presence of nicotine encouraged nematodes to move away from the
302   application site on the Petri dish (Fig. 6D,H).

303   The effects of nicotine on locomotion vary according to dose and over time
304   (Sobkowiak et al., 2011). In other animal models, acute administration of nicotine evokes also
305   dual changes in locomotor activity (Matta et al., 2007). Innate chemosensory preferences are
306   often encoded by sensory neurons that are specialized for attractive or avoidance behaviors.
307   Tsunozaki et al. (2008) show that one olfactory neuron in *Caenorhabditis elegans*, AWC^{ON},

308   has the potential to direct both attraction and repulsion. Attraction, the typical AWC^{ON}

309   behavior, requires a receptor-like guanylate cyclase GCY-28 that acts in adults and localizes

310   to AWC^{ON} axons (Tsunozaki et al., 2008). Interestingly, in our previous research we found

311   GCY-28 only in naïve worms, which were distinctively attracted by nicotine. GCY-28 was

312   absent in the protein complexes involved in response to low and high concentration of

313   nicotine (Sobkowiak et al., 2016). This may suggest that in the presence of nicotine we

314   observed the same kind of switching by presence or absence of food in control nematodes and

315   worms adapted to low nicotine concentration (Fig. 4D).

316   Nicotine induces profound behavioral responses in *C. elegans* that mimic those

317   observed in mammals. The genes and pathways regulating nicotine dependence in mammals

318   are functionally conserved in *C. elegans*, including nicotinic acetylcholine receptors

319   (nAChRs, the molecular target of nicotine) and serotonin and dopamine-mediated

320   neurotransmission. In this study, the exposure started from hatching and lasted 55 h, i.e. at

321   least 3/4 of the *C. elegans* life cycle. Sellings et al. (2013) demonstrated, and we confirmed

322   this finding, that *C. elegans* exhibits motivational behavior patterns towards nicotine, similar

323   to those observed in mammalian models. The motivational behavior pattern is modulated, as

324   we show, by food.

325   We revealed that nicotine can reduce worm body size (Fig. 3). The anorectic effects of

326   smoking have been well documented in human subjects, and the principal reason cited by

327   female teenagers for why they smoke is weight control. On average, smokers weigh 5 kg less

328   than nonsmokers and have significantly lower body mass index than nonsmokers. Similarly,

329   nicotine decreases feeding in animal models, suggesting that nicotine in tobacco is important

330   for the effects of smoking on appetite (Picciotto and Kenny, 2013).

331   Our results confirm that *C. elegans* may serve as a useful model organism for nicotine-

332   motivated and food-motivated behaviors that could help in understanding the mechanisms

333   underlying the anorectic effects of taking nicotine. Thus research on *C. elegans* may facilitate

334   the development of novel treatments to help with smoking cessation and with preventing or

335   treating obesity.

336

337

338

339

340

341 **4   Materials and methods**

342 **4.1   *Caenorhabditis elegans* maintenance**

343   All tests were performed on the wild-type Bristol N2 strain of *C. elegans* obtained from
344   the Caenorhabditis Genetics Center (CGC) at the University of Minnesota (Duluth,
345   Minnesota, USA). Standard methods were used for the maintenance and manipulation of
346   strains (Stiernagle, 2006). Nematodes were kept at 22°C on nematode growth medium (NGM)
347   agar plates seeded with *Escherichia coli* strain OP50 as a source of food according to a
348   standard protocol (Brenner, 1974). Chunks of mixed-stage starved worms were transferred
349   onto enriched nematode growth (ENG) plates (50 mm in diameter) seeded with bacterial food
350   (*E coli* OP50), and the worms were allowed to grow for about 5 days at 22°C (Fig. 1A).

351

352 **4.2   Chronic exposure of *C. elegans* to uniform nicotine concentration**

353   (-)-Nicotine (free base) was obtained from Sigma-Aldrich. The drug was added
354   directly to the NGM medium and allowed to diffuse throughout the medium for 48–72 h to
355   ensure a uniform concentration.

356   To minimize interaction between *E. coli* and nicotine, 10 drops of liquid culture of *E.*
357   *coli* OP50 were added just before chronic exposure of *C. elegans* to nicotine on agar plates.
358   Bacteria were evenly distributed by a spreader on the surface of the NGM medium, and the
359   plates with the lids removed were left to dry for about 10 min. The short drying of the agar
360   surface enabled the nematodes free movement and proper nutrition.

361   When the culture of nematodes contained mostly adults able to lay eggs, chunks of the
362   agar with nematodes were transferred onto the plates for chronic exposure to a uniform
363   nicotine concentration (0.01 M or 1 mM, Fig. 1B). In the controls, the chunks were
364   transferred to nicotine-free NGM plates. To synchronize the worms, parent worms were
365   allowed to lay eggs for 3 h and next the adults were removed. The remaining eggs were
366   incubated for 55 h at 22°C to adulthood (Altun and Hall, 2009). The dosages were selected
367   based on our preliminary study as well previous reports (Feng et al., 2006; Matta et al., 2007;
368   Sobkowiak et al., 2011; Waggoner et al., 2000), in which nicotine treatment had a biphasic
369   response.

4.3 Measurement of worm size

The measurements of worm size and the behavioral nicotine gradient experiments were performed using adult hermaphrodites, which were kept in the presence of nicotine from hatching to adulthood. Body size of adult worms was estimated in control conditions (0 mM nicotine) and after 55 h exposure to 0.01 and 1 mM nicotine. To measure body length, volume, and surface area, the worms were put on an NGM plate, and pictures were taken using a stereomicroscope and analyzed using WormSizer, as previously described (Moore et al., 2013). WormSizer is open-source software that is useful for detecting relatively subtle phenotypes and morphological changes that may have been difficult to assess upon visual inspection. Eight biological replicates, typically including 12 individuals per experimental variant, were analyzed.

4.4 Behavioral nicotine gradient assay

In all the experiments we used young adult worms exposed to 0 mM (control), 0.01 mM, and 1 mM nicotine. After 55 h of nicotine exposure, single young adult hermaphrodites from each adaptation variant were transferred to assay plates containing NGM medium with 4 treatment variants (Fig. 1C):

(1) no food, no radial Gaussian-shaped nicotine gradient (F-n);

(2) no food, a radial Gaussian-shaped nicotine gradient (F-N);

(3) spatially uniform concentration of food, no radial Gaussian-shaped nicotine gradient (F+n);

(4) spatially uniform concentration of food, a radial Gaussian-shaped nicotine gradient (F+N).

The behavioral nicotine gradient assays were performed in standard Petri dishes (inner diameter 92 mm) containing 5.8 mL of NGM. In variants 3 and 4, NGM agar was seeded with 3 drops of the same medium with *E. coli* OP50, while in variants 1 and 2 the plates were seeded with 3 drops of sterile medium without *E. coli* (i.e. without food), and allowed to desiccate for about 10 min. The radial Gaussian-shaped nicotine gradient was formed by adding 1 µL of 580 mM nicotine solution in water at the center of the plate just before the tracking. Nicotine concentration was estimated in the range of 0-580 mM, according to the diffusion equation for a point bolus in a thin slab (Crank, 1975) (Fig. 2B).

For each assay plate, time was recorded for estimation of nicotine concentration during the assay. At each time point of the assay, the concentration of nicotine (mM) at the position of the worm was estimated according to the solution of the diffusion equation (Crank, 1975)

14

402 for a point bolus in a cylindrical, aqueous volume having the same dimensions as the agar in

403 the assay plate (diameter 9.2 cm; depth 0.1 cm). Exact nicotine concentration (in mM) was

404 calculated by the solution of Fick's equation for 2-dimensional diffusion with no border

405 (Crank, 1975; Iino and Yoshida, 2009) .

406
$$C = \frac{N_0 e^{-\frac{r^2}{400Dt}}}{4\Pi dDt}$$

407 The Fick's equation for 2-dimensional diffusion with no border: C = nicotine concentration

408 [mM]; $N_0 = 0.58$ [mmole] is the number of moles of nicotine spotted (1 μL of 580 mM

409 nicotine contains 0.58 mmoles of nicotine); $D = 0.000\ 000\ 42$ [cm^2/s] is the diffusion

410 coefficient of nicotine (estimated basing on Sellings et al., 2013); $d = 0.1$ [cm] is the depth of

411 the agar; $t =$ is the time [s] after spotting nicotine at the center of the plate; $r =$ is the distance

412 [cm] between the peak of the gradient and the location of the animal (Crank, 1975; Iino and

413 Yoshida, 2009).

414

415   To study the behavior of worms, we used an automated tracking system to follow

416 individual young adults crawling on NGM plates (Kowalski et al., 2014). Single young adults

417 (aged ~ 55 h) were manually picked off an adaptation plate (Fig. 1B) and placed using a

418 platinum pick in a 1-μL droplet of water on the agar surface of the gradient assay plate (Fig.

419 1C), 15 mm from the plate center (Fig. 2A). Putting a worm in a droplet of water is an

420 effective method for rapid transferring of a single animal without scratching the agar surface

421 (important for obtaining high-contrast videos and perfect tracking) and for testing the

422 condition of the worm (injured nematodes, which could not properly swim in a drop of water,

423 were rejected). The surface tension of the drop of water prevented the nematode from

424 creeping out. Next, the plate was placed in a device for tracking nematodes. Observation of

425 the nematode allowed us to notice the moment when the 1-μL drop disappeared by

426 evaporation/absorption in agar and released the worm, which could then freely move on the

427 plate. At that moment, in the center of the plate, 1 μL of 580 mM nicotine or 1 μL of H$_2$O was

428 spotted (Fig. 1C and 2A). Worm tracking began no more than 10 s after the application of

429 nicotine or water in the center of the plate. Each worm was tracked for 3600 s or until it

430 reached the edge of the plate.

431 4.5 Worm tracking system

432   Custom worm tracker software (WormSpy) was used to move the camera
433   automatically to re-center the worm under the field of view during recording (Kowalski et al.,
434   2014). The automated tracking system comprises a stereomicroscope (Olympus SZ11), a
435   modified (with unscrewed lens) web camera (Logitech QuickCam Pro 9000) with 640×480
436   resolution to acquire worm videos, and a desktop PC running under Windows 7. The tracking
437   system located the worm's centroid (defined as the geometrical center of the smallest
438   rectangle that could be drawn around the worm) and recorded its x and y coordinates with a
439   sampling rate of 2 s^{-1}. When a worm neared the edge of the field of view, the tracking system
440   automatically re-centered the worm by moving the stage and recorded the distance that the
441   stage was moved. We reduced the variation in sampling rate as a consequence of the small
442   differences in the time it took to re-center the worm and the need to take data only when the
443   stage was stationary by developing a simultaneous localization and tracking method for a
444   worm tracking system (Kowalski et al., 2014). The spatiotemporal track of each worm was
445   reconstructed from the record of centroid locations and camera displacements. The
446   instantaneous speed and trajectory were computed using the displacement of the centroid in
447   successive samples. The tracking system recorded the worm's position, speed, distance from
448   the center of the plate and from the starting point, estimated nicotine concentration in the
449   surroundings the worm, and trajectory at 0.5-s intervals. Individual worms moved away from
450   their starting location, leaving complex tracks. Video recordings were carried out at room
451   temperature (22°C). Five independent experiments were performed per dosage group.

452   All the experimental procedures presented in this paper were in compliance with the
453   European Communities Council Directive of 24 November 1986 (86/609/EEC).

454 4.6 Data analysis and statistical analysis

455   The measurements from experiments were pooled for each treatment variant and the
456   median values were calculated. The data were not normally distributed, as determined by the
457   Shapiro-Wilk W-test and Kolmogorov-Smirnov & Lilliefors method. Due to this, the
458   Kruskal–Wallis tests followed by Dunn's multiple comparison post-hoc tests were performed.
459   Statistical significance was considered at $p < 0.05$. The calculations and graphs were done by
460   using Statistica software (StatSoft, Inc., Tulsa, Oklahoma, USA).

461   Worm body size data are presented as mean±S.D. of at least 8 independent

462   experiments. One-way ANOVA followed by Scheffe's test were performed to determine

463   statistical differences between groups with the aid of Statistica (StatSoft software).

464   Significance was set at $p < 0.05$.

465

466

Acknowledgments

467

468   We would like to thank the Caenorhabditis Genetics Center, which is funded by the

469   NIH National Center for Research Resources (NCRR), for supplying the nematode strain used

470   in this research. We thank also Sylwia Ufnalska, an author's editor, for help in improving the

471   manuscript. This study was supported by funds from the Department of Cell Biology, Faculty

472   of Biology, Adam Mickiewicz University (Poznań, Poland).

473

474

5   References

Altun, Z.F., Hall, D.H. 2016. Handbook of *C. elegans* anatomy. In WormAtlas. http://www.wormatlas.org/hermaphrodite/hermaphroditehomepage.htm

Bargmann, C.I., 2006. Chemosensation in *C. elegans*, in: Community, T.C.e.R. (Ed.) WormBook. WormBook.

Benowitz, N.L., 2010. Nicotine addiction. N Engl J Med 362(24), 2295-2303.

Benowitz, N.L., Hukkanen, J., Jacob, P., 3rd, 2009. Nicotine chemistry, metabolism, kinetics and biomarkers. Handb Exp Pharmacol 192(192), 29-60.

Brenner, S., 1974. The genetics of *Caenorhabditis elegans*. Genetics 77(1), 71-94.

Chase, D.L., Koelle, M.R., 2007. Biogenic amine neurotransmitters in *C. elegans*, in: Community, T.*C.e*.R. (Ed.) Wormbook.

Crank, J., 1975. The mathematics of diffusion. Oxford: Clarenden.

de Bono, M., Bargmann, C.I., 1998. Natural variation in a neuropeptide Y receptor homolog modifies social behavior and food response in *C. elegans*. Cell 94(5), 679-689.

Feng, Z., Li, W., Ward, A., Piggott, B.J., Larkspur, E.R., Sternberg, P.W., Xu, X.Z., 2006. A *C. elegans* model of nicotine-dependent behavior: regulation by TRP-family channels. Cell 127(3), 621-633.

Filozof, C., Fernandez Pinilla, M.C., Fernandez-Cruz, A., 2004. Smoking cessation and weight gain. Obes Rev 5(2), 95-103.

Grana, R., Benowitz, N., Glantz, S.A., 2014. E-cigarettes: a scientific review. Circulation 129(19), 1972-1986.

Gray, J.M., Hill, J.J., Bargmann, C.I., 2005. A circuit for navigation in *Caenorhabditis elegans*. Proc Natl Acad Sci U S A 102(9), 3184-3191.

Iino, Y., Yoshida, K., 2009. Parallel use of two behavioral mechanisms for chemotaxis in *Caenorhabditis elegans*. J Neurosci 29(17), 5370-5380.

Jo, Y.H., Talmage, D.A., Role, L.W., 2002. Nicotinic receptor-mediated effects on appetite and food intake. J Neurobiol 53(4), 618-632.

Kowalski, M., Kaczmarek, P., Kabaciński, R., Matuszczak, M., Tranbowicz, K., Sobkowiak, R., 2014. A simultaneous localization and tracking method for a worm tracking system. International Journal of Applied Mathematics and Computer Science 24(3), 599–609.

Liu, R.H., Mizuta, M., Matsukura, S., 2004. The expression and functional role of nicotinic acetylcholine receptors in rat adipocytes. J Pharmacol Exp Ther 310(1), 52-58.

508 **Matsuura, T., Miura, H., Nishino, A.,** 2013. Inhibition of gustatory plasticity due to acute
509   nicotine exposure in the nematode *Caenorhabditis elegans*. Neurosci Res 77(3), 155-161.

510 **Matta, S.G., Balfour, D.J., Benowitz, N.L., Boyd, R.T., Buccafusco, J.J., Caggiula, A.R.,**
511   **Craig, C.R., Collins, A.C., Damaj, M.I., Donny, E.C., Gardiner, P.S., Grady, S.R.,**
512   **Heberlein, U., Leonard, S.S., Levin, E.D., Lukas, R.J., Markou, A., Marks, M.J.,**
513   **McCallum, S.E., Parameswaran, N., Perkins, K.A., Picciotto, M.R., Quik, M., Rose,**
514   **J.E., Rothenfluh, A., Schafer, W.R., Stolerman, I.P., Tyndale, R.F., Wehner, J.M.,**
515   **Zirger, J.M.,** 2007. Guidelines on nicotine dose selection for *in vivo* research.
516   Psychopharmacology (Berl) 190(3), 269-319.

517 **McKay, J.P., Raizen, D.M., Gottschalk, A., Schafer, W.R., Avery, L.,** 2004. eat-2 and eat-
518   18 Are Required for Nicotinic Neurotransmission in the *Caenorhabditis elegans* Pharynx.
519   Genetics 166(1), 161-169.

520 **Mineur, Y.S., Abizaid, A., Rao, Y., Salas, R., DiLeone, R.J., Gundisch, D., Diano, S., De**
521   **Biasi, M., Horvath, T.L., Gao, X.B., Picciotto, M.R.,** 2011. Nicotine decreases food
522   intake through activation of POMC neurons. Science 332(6035), 1330-1332.

523 **Moore, B.T., Jordan, J.M., Baugh, L.R.,** 2013. WormSizer: high-throughput analysis of
524   nematode size and shape. PLoS ONE 8(2), e57142.

525 **Nichter, M., Vuckovic, N., Tesler, L., Adrian, S., Ritenbaugh, C.,** 2004. Smoking as a
526   weight-control strategy among adolescent girls and young women: a reconsideration. Med
527   Anthropol Q 18(3), 305-324.

528 **Picciotto, M.R., Kenny, P.J.,** 2013. Molecular mechanisms underlying behaviors related to
529   nicotine addiction. Cold Spring Harb Perspect Med 3(1), a012112.

530 **Pierce-Shimomura, J.T., Morse, T.M., Lockery, S.R.,** 1999. The fundamental role of
531   pirouettes in *Caenorhabditis elegans* chemotaxis. J Neurosci 19(21), 9557-9569.

532 **Ramot, D., Johnson, B.E., Berry, T.L., Jr., Carnell, L., Goodman, M.B.,** 2008. The
533   Parallel Worm Tracker: a platform for measuring average speed and drug-induced
534   paralysis in nematodes. PLoS ONE 3(5), e2208.

535 **Rose, J.K., Miller, M.K., Crane, S.A., Hope, K.A., Pittman, P.G.,** 2013. Parental and larval
536   exposure to nicotine modulate spontaneous activity as well as cholinergic and GABA
537   receptor expression in adult *C. elegans*. Neurotoxicol Teratol.

538 **Sawin, E.R., Ranganathan, R., Horvitz, H.R.,** 2000. *C. elegans* locomotory rate is
539   modulated by the environment through a dopaminergic pathway and by experience through
540   a serotonergic pathway. Neuron 26(3), 619-631.

541 **Schuller, H.M.,** 2009. Is cancer triggered by altered signaling of nicotinic acetylcholine
542   receptors? Nat Rev Cancer 9(3), 195-205.

543 **Sellings, L., Pereira, S., Qian, C., Dixon-McDougall, T., Nowak, C., Zhao, B., Tyndale,**
544   **R.F., van der Kooy, D.,** 2013. Nicotine-motivated behavior in *Caenorhabditis elegans*
545   requires the nicotinic acetylcholine receptor subunits acr-5 and acr-15. Eur J Neurosci.

546 **Sobkowiak, R., Kowalski, M., Lesicki, A.,** 2011. Concentration- and time-dependent
547   behavioral changes in *Caenorhabditis elegans* after exposure to nicotine. Pharmacol
548   Biochem Behav 99, 365–370.

549 **Sobkowiak, R., Lesicki, A.,** 2011. Komórkowe szlaki sygnalizacyjne aktywowane przez
550   nikotynę. [Cell signaling pathways activated by nicotine]. Post Biol. Kom 38(4), 581-596.

551 **Sobkowiak, R., Lesicki, A.,** 2013. Wchłanianie, przemiany metaboliczne i wydalanie
552   nikotyny u czlowieka. [Absorption, metabolism and excretion of nicotine in humans]. Post
553   Biochem 59(1), 33-44.

554 **Sobkowiak, R., Zieleziński, A., Wojciech M Karlowski, W.M., Lesicki, A.,** 2017. Nicotine
555   affects protein complex rearrangement in *Caenorhabditis elegans* cells. Drug Chem
556   Toxicol, DOI: 10.1080/01480545.2016.1264411

557 **Stiernagle, T.,** 2006. Maintenance of *C. elegans*, in: Community, T.C.e.R. (Ed.) WormBook.
558   WormBook.

559 **Tsunozaki, M., Chalasani, S.H., Bargmann, C.I.,** 2008. A behavioral switch: cGMP and
560   PKC signaling in olfactory neurons reverses odor preference in *C. elegans*. Neuron 59(6),
561   959-971.

562 **Waggoner, L.E., Dickinson, K.A., Poole, D.S., Tabuse, Y., Miwa, J., Schafer, W.R.,** 2000.
563   Long-term nicotine adaptation in *Caenorhabditis elegans* involves PKC-dependent
564   changes in nicotinic receptor abundance. J Neurosci 20(23), 8802-8811.

565 **[WHO] World Health Organization,** 2012. WHO global report: mortality attributable to
566   tobacco.
567   http://www.who.int/tobacco/publications/surveillance/rep_mortality_attributable/en/.

568 **Wolf, F.W., Heberlein, U.,** 2003. Invertebrate models of drug abuse. J Neurobiol 54(1), 161-
569   178.

570

571 **Figure 1.** Outline of experimental procedures. (**A**) *Incubation. Caenorhabditis elegans* were

572 grown for 5 days at 22°C on ENG medium plates spread with *E. coli.* (**B**) *Long-term nicotine*

573 *adaptation*. Adult worms were transferred to NGM plates seeded with food, by transferring of

574 chunks of the medium with nematodes onto control plates (0 mM nicotine) and adaptation

575 plates containing 0.01 mM nicotine (low nicotine concentration) or 1 mM nicotine (high

576 nicotine concentration). All adult worms after 3 h were removed and only the laid eggs were

577 left. Incubation at 22°C for 55 h. (**C**) *Transfer of single adult nematodes onto gradient assay*

578 *plates*. Duration of the experiment: 3600 s. White plate = no food; uniform gray plate =

579 presence of food; peak at the plate center = application point of nicotine at the beginning of

580 the experiment; no peak at the plate center = control plates (with water instead of nicotine).

581 (**D**) Data analysis of used behavioral endpoints measured in the tests.

582

583 **Figure 2.** (**A**) Configuration of the plate for nicotine gradient assay of *Caenorhabditis*

584 *elegans*. One worm was placed in a 1-μL drop of water and thus trapped for several dozen

585 seconds in the start position of a Petri dish containing 5.8 mL of NGM medium. Immediately

586 after the worm was released, nicotine was spotted into the center of the plate (peak). Water

587 was applied instead of nicotine in control variants. (**B**) Theoretical distribution of nicotine

588 concentration depending on the distance (r = radius) from the peak. The gradient was formed

589 by placing 1 μL of 580 mM (-)-nicotine at the center of the plate at the beginning of

590 experiment. Nicotine appears to move smoothly and systematically from high-concentration

591 areas to low-concentration areas, following Fick's laws. Concentration estimates were made

592 as described in section 2.4 (see the equation)

593

594 **Figure 3.** Differences in body size of *Caenorhabditis elegans* after 55 h of growth in control

595 conditions (0 mM nicotine) and in the presence of 0.01 mM nicotine and 1 mM nicotine

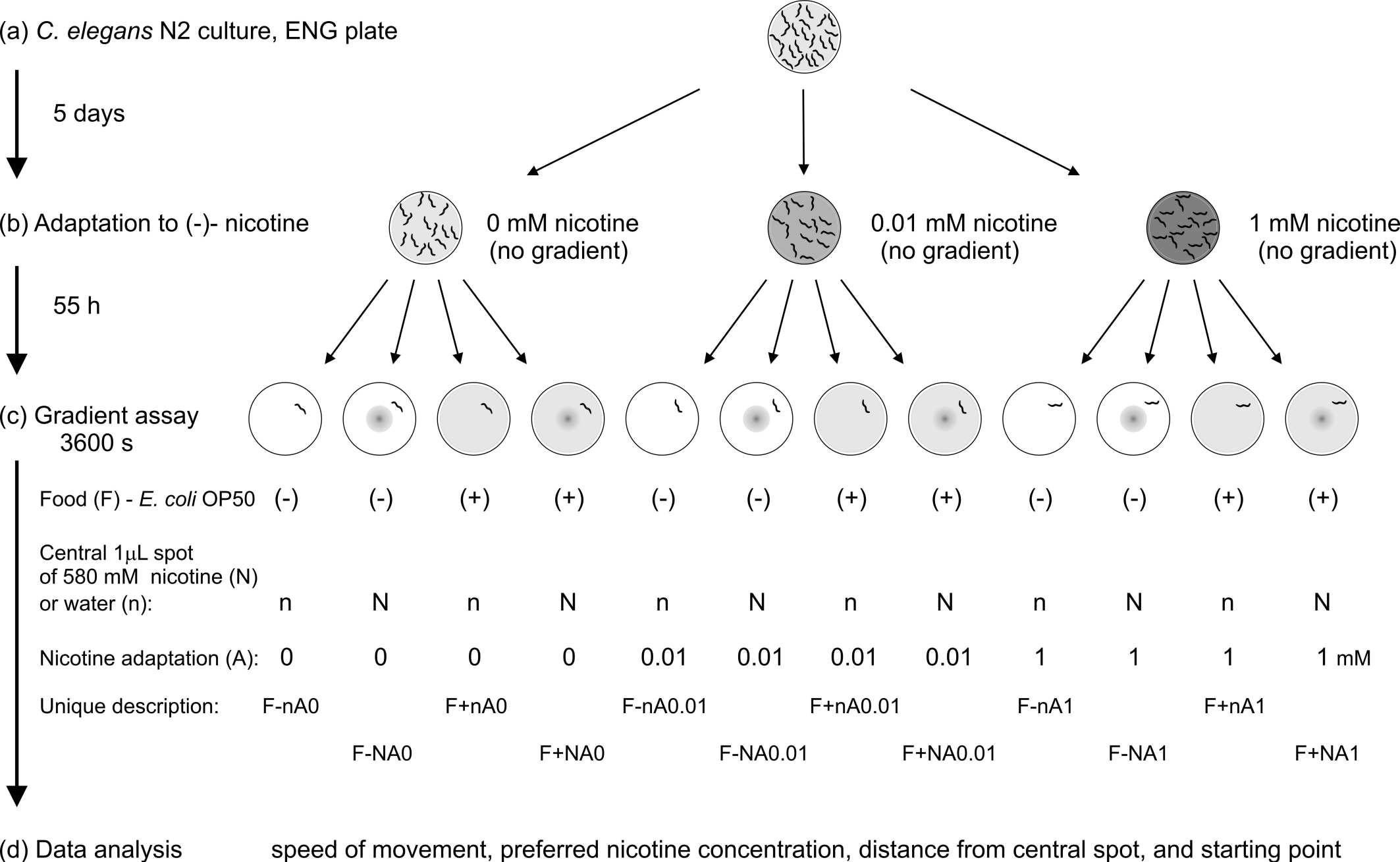596 ($*p < 0.05$, Scheffe test, $N = 96$ at least).

597

598 **Figure 4.** Average values of factors describing the behavior of *Caenorhabditis elegans*: (**A**)

599 speed of movement; (**B**) distance from the start position; (**C**) distance from the peak; (d)

600 preferred nicotine concentration; "ns" denoted no statistically significant differences between

601 experimental conditions. Other groups were statistically significantly different from each

602 other ($p < 0.05$, Kruskal-Wallis ANOVA by ranks test, data pooled from 5 independent

603 experiments, $N = 14\ 370$).

604 **Figure 5.** Locomotor activity (centroid speed) of *Caenorhabditis elegans* in the tested
605 experimental variants. The data are medians and 25th and 75th percentiles of 5 pooled
606 experiments. F-/F+ = absence/presence of food, respectively; n = no nicotine gradient; N =
607 presence of nicotine gradient; A0 = naïve worms, which never had contact with nicotine
608 before; A0.01 = worms adapted to 0.01 mM nicotine; A1 = worms adapted to 1 mM nicotine.

609

610 **Figure 6.** Distance from the start position of *Caenorhabditis elegans* in the tested
611 experimental variants. The data are medians and 25th and 75th percentiles of 5 pooled
612 experiments. F-/F+ = absence/presence of food, respectively; n = no nicotine gradient; N =
613 presence of nicotine gradient; A0 = naïve worms, which never had contact with nicotine
614 before; A0.01 = worms adapted to 0.01 mM nicotine; A1 = worms adapted to 1 mM nicotine.

615

616 **Figure 7.** Time-dependent changes in distance from the peak of *Caenorhabditis elegans* in
617 the tested experimental variants. The data are medians and 25th and 75th percentiles of 5
618 pooled experiments. F-/F+ = absence/presence of food, respectively; n = no nicotine gradient;
619 N = presence of nicotine gradient; A0 = naïve worms, which never had contact with nicotine
620 before; A0.01 = worms adapted to 0.01 mM nicotine; A1 = worms adapted to 1 mM nicotine.

621

622 **Figure 8.** Time-dependent changes in nicotine concentration preferred by *Caenorhabditis*
623 *elegans* in the tested experimental variants. The data are medians and 25th and 75th
624 percentiles of 5 experiments. F-/F+ = absence/presence of food, respectively; n = no nicotine
625 gradient; N = presence of nicotine gradient; A0 = naïve worms, which never had contact with
626 nicotine before; A0.01 = worms adapted to 0.01 mM nicotine; A1 = worms adapted to 1 mM
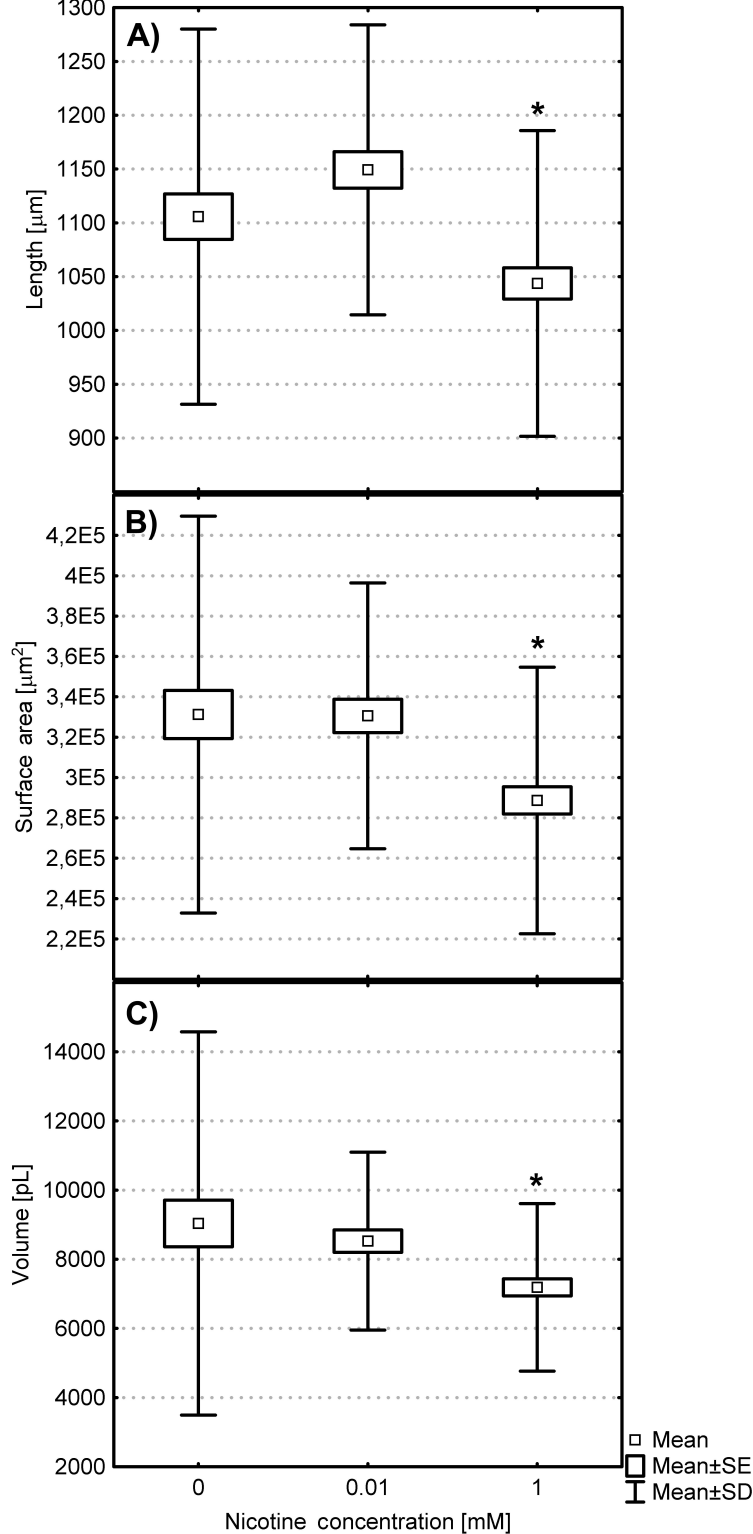627 nicotine.

23

Figure 1. Outline of experimental procedures. (A) Incubation. *Caenorhabditis elegans* were grown for 5 days at 22°C on ENG medium plates spread with E. coli. (B) Long-term nicotine adaptation. Adult worms were transferred to NGM plates seeded with food, by transferring of chunks of the medium with nematodes onto control plates (0 mM nicotine) and adaptation plates containing 0.01 mM nicotine (low nicotine concentration) or 1 mM nicotine (high nicotine concentration). All adult worms after 3 h were removed and only the laid eggs were left. Incubation at 22°C for 55 h. (C) Transfer of single adult nematodes onto gradient assay plates. Duration of the experiment: 3600 s. White plate = no food; uniform gray plate = presence of food; peak at the plate center = application point of nicotine at the beginning of the experiment; no peak at the plate center = control plates (with water instead of nicotine). (D) Data analysis of used behavioral endpoints measured in the tests.
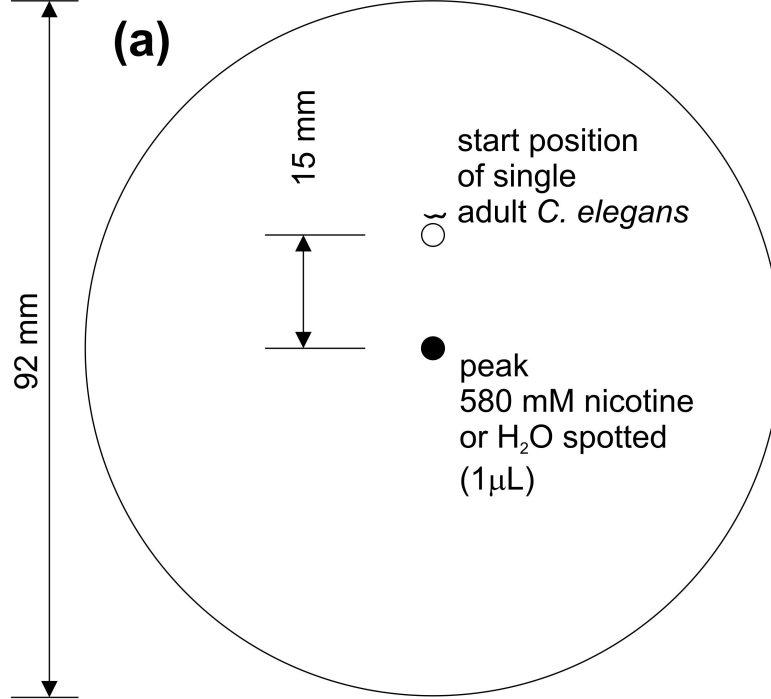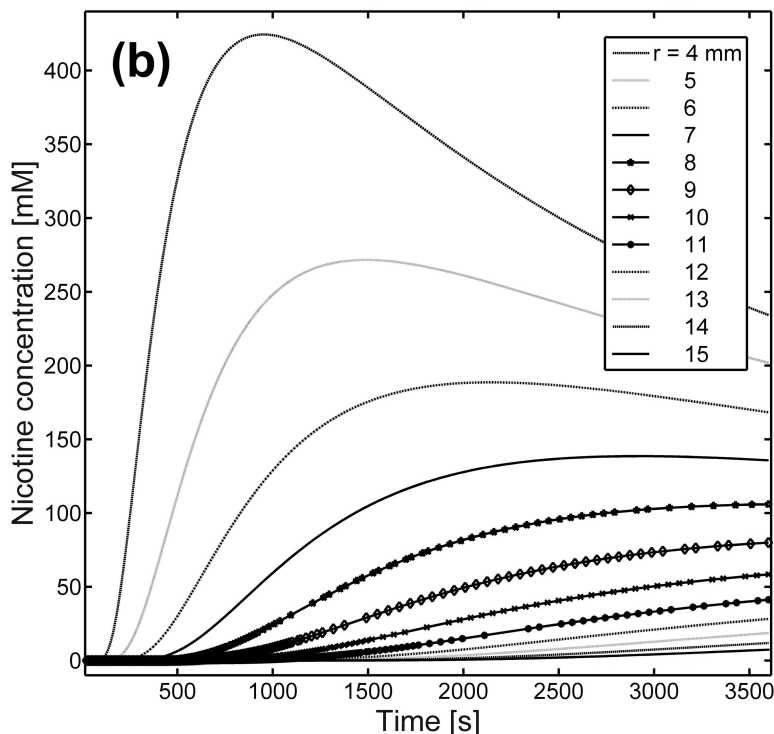
Figure 2. (A) Configuration of the plate for nicotine gradie nt assay of *Caenorhabditis elegans*. One worm was placed in a 1-⊠L drop of water and thus trapped for several dozen seconds in the start position of a Petri dish containing 5.8 mL of NGM medium. Immediately after the worm was released, nicotine was spotted into the center of the plate (peak). Water was applied instead of nicotine in control variants. (B) Theoretical distribution of nicotine concentration depending on the distance (r = radius) from the peak. The gradient was formed by placing 1 ⊠L of 580 mM (-)-nicotine at the center of the plate at the beginning of experiment. Nicotine appears to move smoothly and systematically from high-concentration areas to low-concentration areas, following Fick's laws. Concentration estimates were made as described in section 2.4 (see the equation).

Figure 3. Differences in body size of *Caenorhabditis elegans* after 55 h of growth in control conditions (0 mM nicotine) and in the presence of 0.01 mM nicotine and 1 mM nicotine (* p < 0.05, Scheffe test, N = 96 at least).
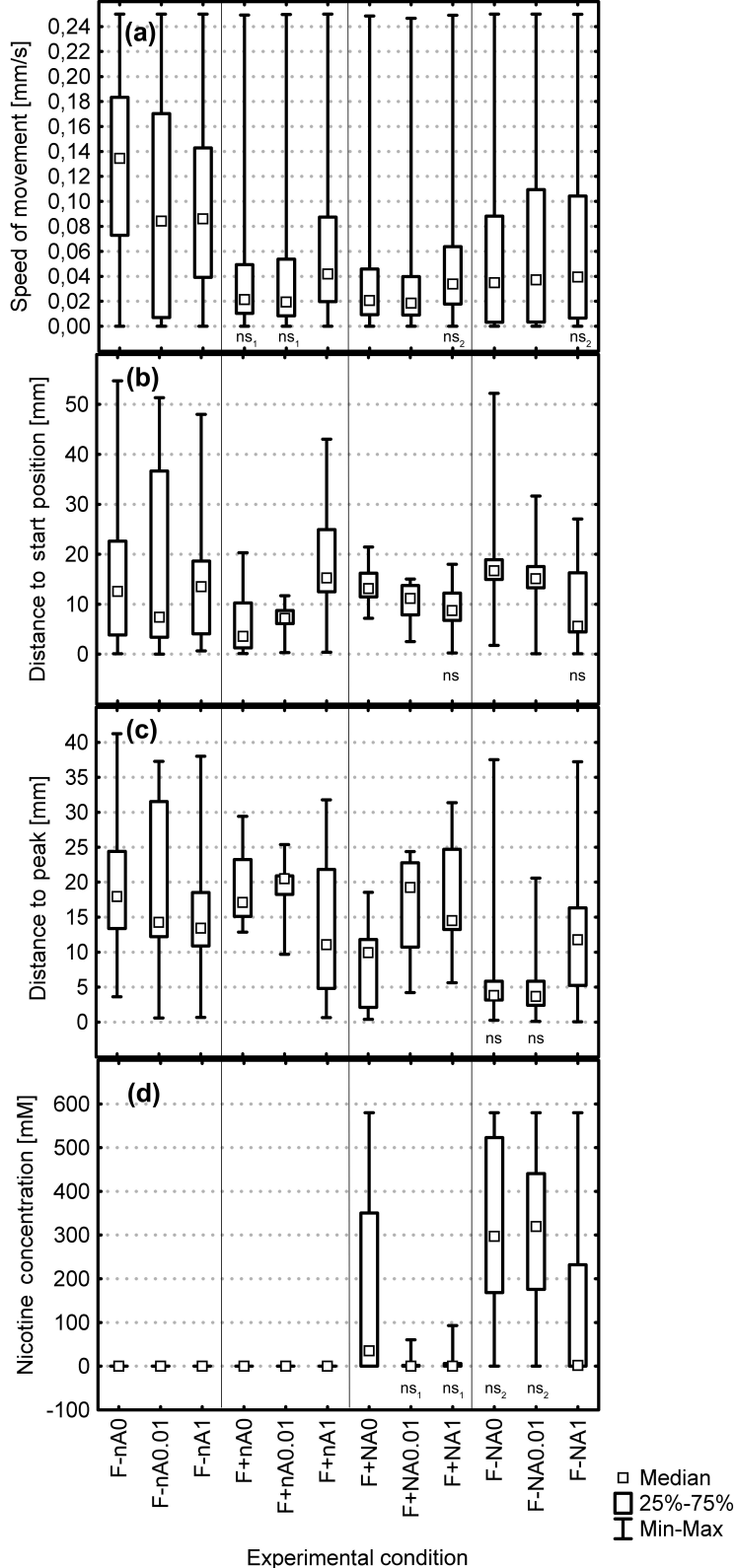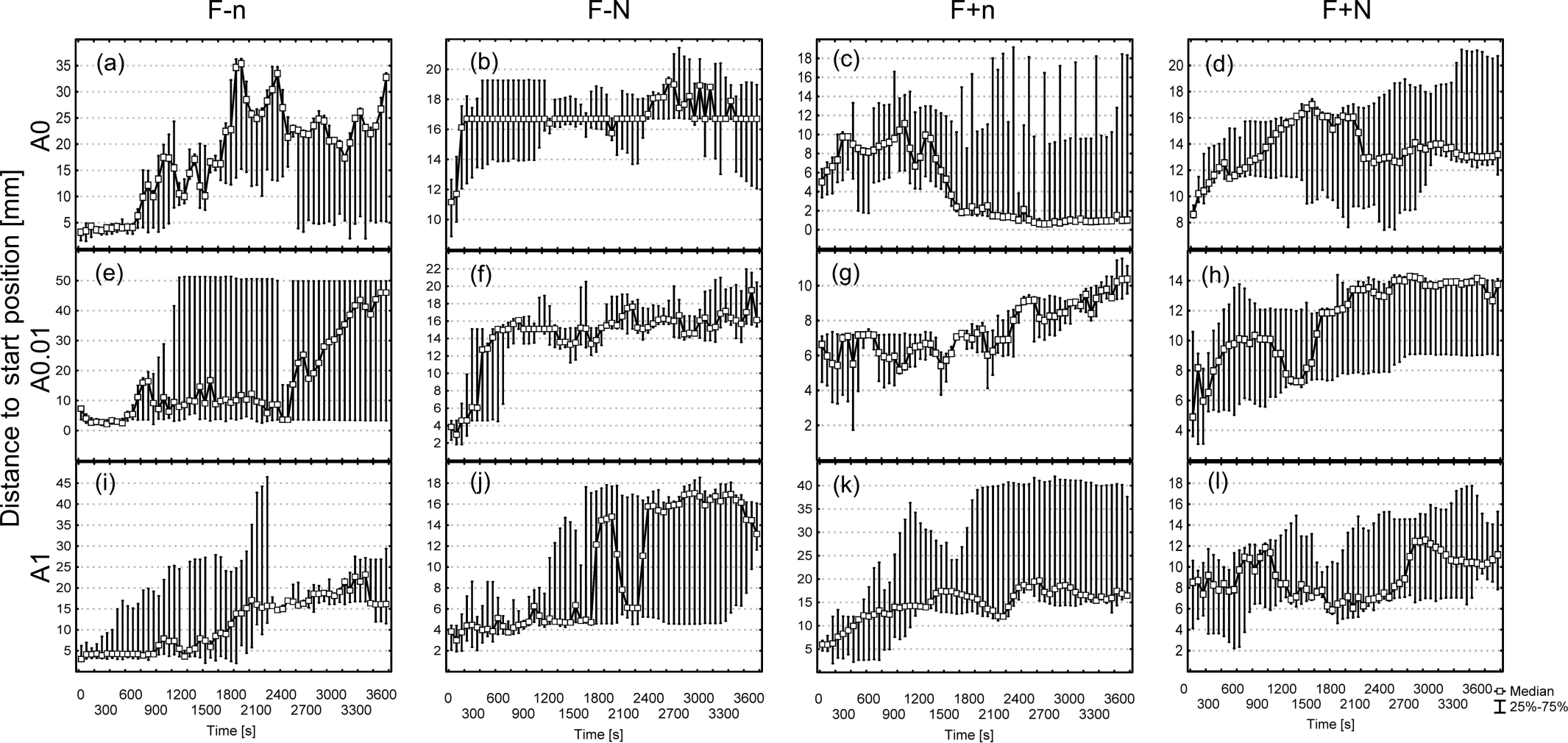
Figure 4. Average values of factors describing the behavior of *Caenorhabditis elegans*: (A) speed of movement; (B) distance from the start position; (C) distance from the peak; (d) preferred nicotine concentration; "ns" denoted no statistically significant differences between experimental conditions. Other groups were statistically significantly different from each other ($p < 0.05$, Kruskal-Wallis ANOVA by ranks test, data pooled from 5 independent experiments, N = 14 370).
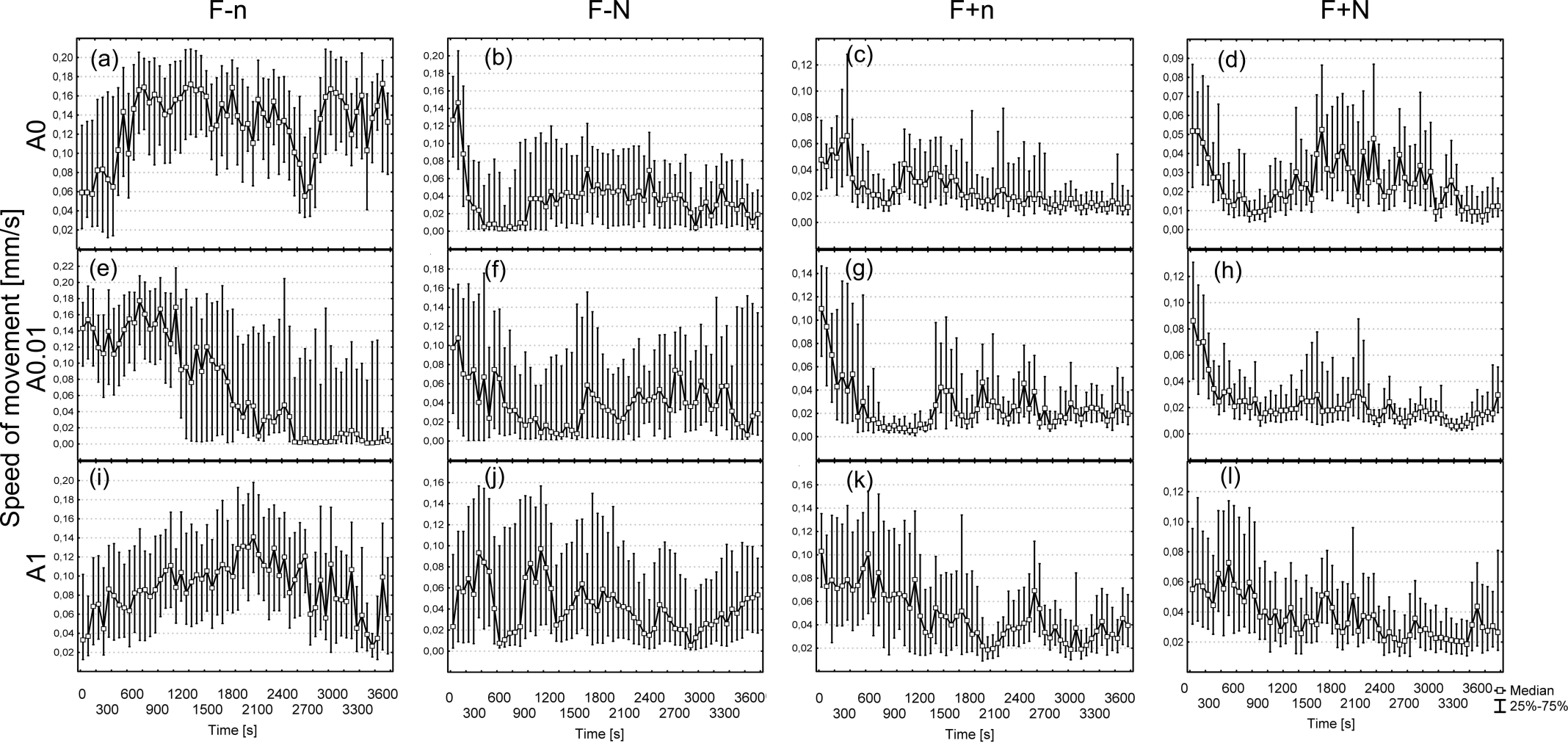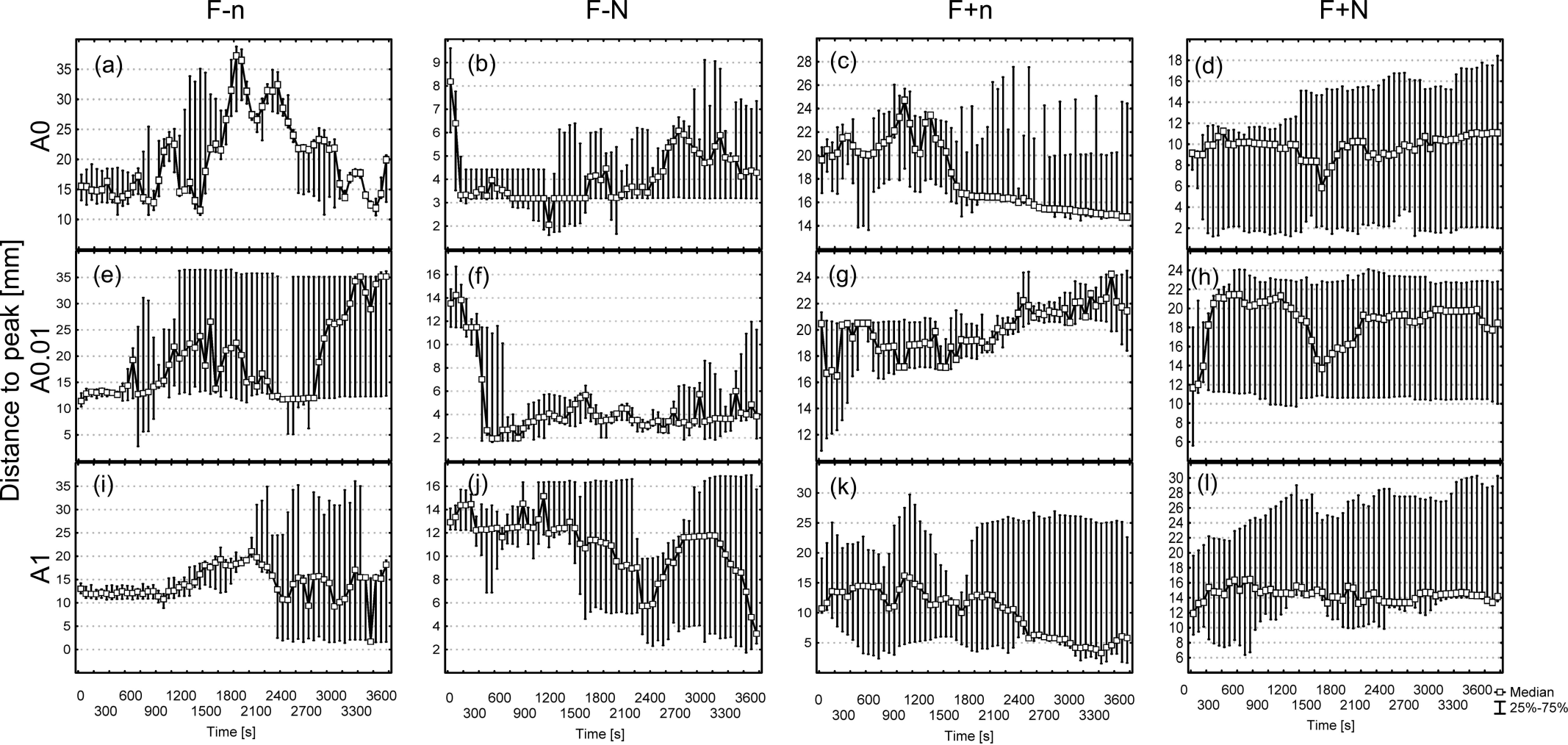
Figure 5. Locomotor activity (centroid speed) of *Caenorhabditis elegans* in the tested experimental variants. The data are medians and 25th and 75th percentiles of 5 pooled experiments. F-/F+ = absence/presence of food, respectively; n = no nicotine gradient; N = presence of nicotine gradient; A0 = naïve worms, which never had contact with nicotine before; A0.01 = worms adapted to 0.01 mM nicotine; A1 = worms adapted to 1 mM nicotine.
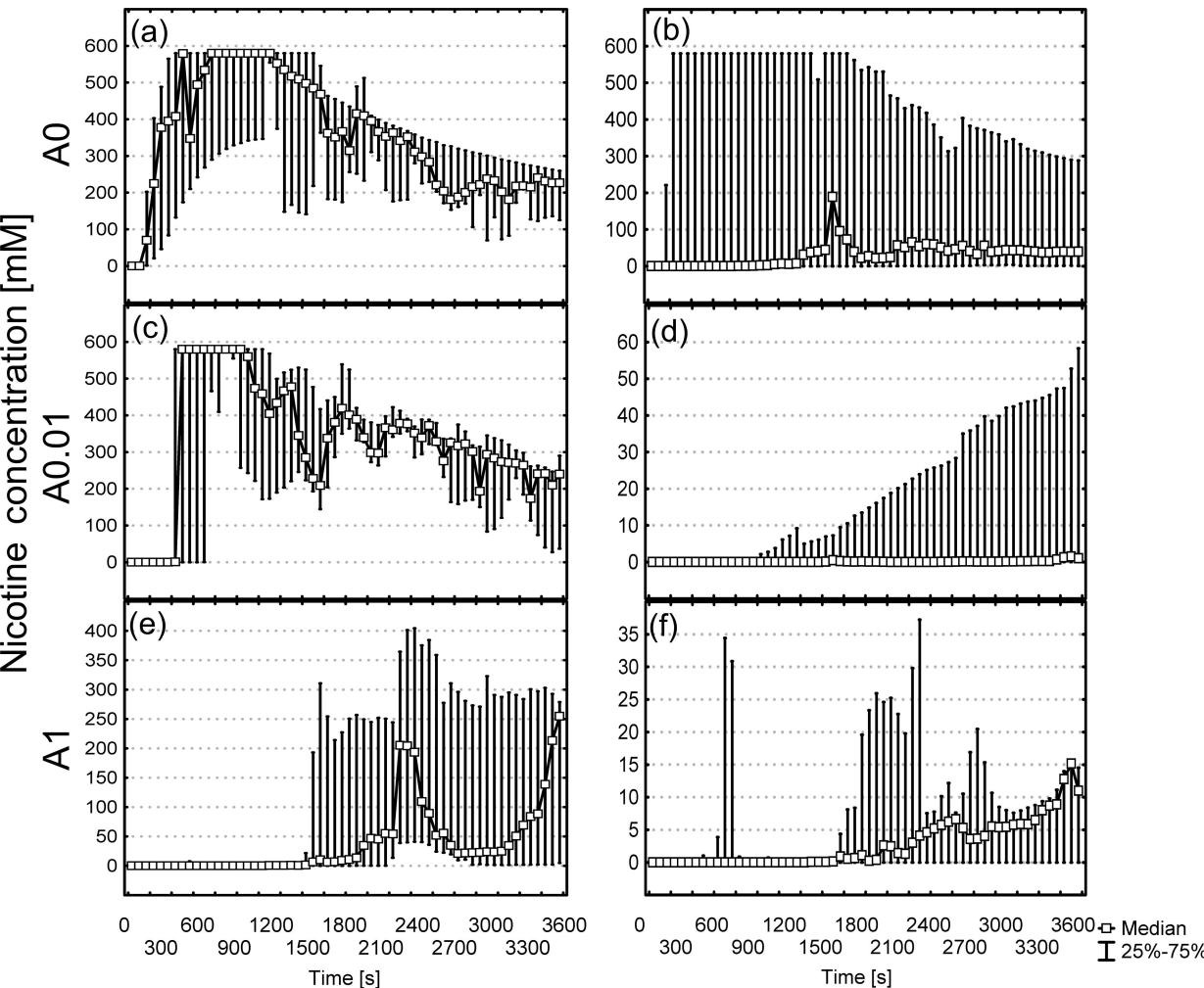
Figure 6. Distance from the start position of *Caenorhabditis elegans* in the tested experimental variants. The data are medians and 25th and 75th percentiles of 5 pooled experiments. F-/F+ = absence/presence of food, respectively; n = no nicotine gradient; N = presence of nicotine gradient; A0 = naïve worms, which never had contact with nicotine before; A0.01 = worms adapted to 0.01 mM nicotine; A1 = worms adapted to 1 mM nicotine.

Figure 7. Time-dependent changes in distance from the peak of *Caenorhabditis elegans* in the tested experimental variants. The data are medians and 25th and 75th percentiles of 5 pooled experiments. F-/F+ = absence/presence of food, respectively; n = no nicotine gradient; N = presence of nicotine gradient; A0 = naïve worms, which never had contact with nicotine before; A0.01 = worms adapted to 0.01 mM nicotine; A1 = worms adapted to 1 mM nicotine.

Figure 8. Time-dependent changes in nicotine concentration preferred by *Caenorhabditis elegans* in the tested experimental variants. The data are medians and 25th and 75th percentiles of 5 experiments. F-/F+ = absence/presence of food, respectively; n = no nicotine gradient; N = presence of nicotine gradient; A0 = naïve worms, which never had contact with nicotine before; A0.01 = worms adapted to 0.01 mM nicotine; A1 = worms adapted to 1 mM nicotine.