Cage-induced stereotypic behaviour in laboratory mice covaries with nucleus accumbens FosB/ΔFosB expression

Danielle Phillips[1], Elena Choleris[1], Kelsy S. J. Ervin[1], Carole Fureix[2], Laura Harper[3], Kathryn Reynolds[4], Lee Niel[5], and Georgia J. Mason[3] *

[1] Department of Psychology and Neuroscience Program, University of Guelph, Ontario
[2] Department of Animal Husbandry, University of Bristol, Langford, UK
[3] Department of Animal Biosciences, University of Guelph, Ontario
[4] Department of Molecular & Cellular Biology, University of Guelph, Ontario
[5] Department of Population Medicine, University of Guelph, Ontario

* Contact for correspondence: gmason@uoguelph.ca

Abstract

Stereotypic behaviour (SB) occurs in certain human disorders (e.g. autism), and animals treated with stimulants or raised in impoverished conditions, including laboratory mice in standard cages. Dysfunctional cortico-basal ganglia pathways have been implicated in these examples, but for cage-induced forms of SB, the relative roles of ventral versus dorsal striatum have not been fully ascertained. Here, we used immunohistochemical staining of FosB and ΔFosB to assess long-term activation within the nucleus accumbens and caudate-putamen of C57BL/6 mice. Housed in typical laboratory cages, these mice spontaneously developed different degrees of route-tracing, bar-mouthing and other forms of SB (spending 0% to over 50% of their active time budgets in this behaviour). The most highly stereotypic mice showed the most elevated FosB/ΔFosB activity in the nucleus accumbens. No such patterns occurred in the caudate-putamen. The cage-induced SB common in standard-housed mice thus involves elevated activity within the ventral striatum, suggesting an aetiology closer to compulsive gambling, eating and drug-seeking than to classic amphetamine stereotypies and other behaviours induced by motor loop over-activation.

Key words: Stereotypic behaviour; stereotypy; abnormal repetitive behaviour; nucleus accumbens; caudate-putamen; striatum; transcription factor; ΔFosB

Stereotypic behaviour (SB) is common in some human neurodevelopmental disorders (e.g. autism), where it seems to reflect dysfunctional basal ganglia circuitry [1, 2]. It can also be experimentally induced in animals by manipulating these circuits [e.g. via stimulants 2, 3]. SB is common, too, in farm, zoo and laboratory animals raised and housed in impoverished enclosures [1, 2, 4]. Here, SB often covaries with generalized signs of impaired behavioural control [1, 4, 5], again suggesting cortico-striatal dysfunction. Furthermore, the types of enclosure that promote SB induce diverse structural and biochemical changes within the basal ganglia [2, 6, 7]. However, more direct evidence for basal ganglia involvement, based on neurological differences that correlate with SB at the individual level, is rare, and comes from just two species: the deer mouse (*Peromyscus maniculatus*) and horse (*Equus caballus*). The deer mouse studies found that animals spontaneously developing high levels of SB, compared to those displaying little or none, have reduced cytochrome oxidase (CO) activity in the ventromedial caudate-putamen (CPu), reduced CO activity in subthalamic nuclei, and altered CPu dynorphin/encephalin ratios consistent with reduced inhibition in cortico-striatal 'motor loops' [2, 6, 7]. Complementary studies highlighted changes in the frontal cortex, including deficient glutathione systems [8]. Equine research, focussing on dopamine receptor densities, has instead revealed elevated D1 and D2 densities within the nucleus accumbens (NAc) of highly stereotypic horses [1], alongside unexpectedly lower D1 densities in the dorso-medial striatum.

Over 30 million mice of the *Mus* genus are used annually in research worldwide, and in standard housing many display SB [9]. Despite this, the neurological bases of laboratory mouse SB are essentially unknown. In one study, the tendencies of two strains, C57BL/6 (henceforth 'C57') and DBA, to develop SB were contrasted, and strain-typical predispositions to high levels suggested to reflect strain differences in the up-regulation of NAc dopamine receptors [3, 10]. Consistently, a second experiment drew parallels between the high SB of C58BL/6 mice compared to other strains, and its enhanced locomotor responses to amphetamine [11]. Neither study, however, looked at within-strain individual differences or obtained strong evidence of causality. A third study revealed distinct individual behavioural correlates of SB in C57s: elevated response repetition in two-choice 'gambling' tasks in the most stereotypic mice [5] (something

other behavioural studies failed to replicate in another strain, CD-1 [12,13]). This effect in the stereotypic C57s was interpreted as reflecting dorsal striatal dysfunction [5]. This summarises what little is known about cage-induced SB in *Mus*. We therefore compared spontaneously high and low SB C57 mice, using immunohistochemistry to assay FosB and ΔFosB. Fos family proteins are transcription factors that regulate gene expression, ΔFosB being a highly stable FosB variant that accumulates over time with repeated stimulation, and that mediates long-term neuronal plasticity [e.g. 14, 15, 16]. ΔFosB thus reflects long–term neuronal activation, in a manner perhaps likely to covary with CO [7]. We investigated whether, within the basal ganglia, the caudate-putamen and/or the nucleus accumbens show evidence of sustained activation in highly stereotypic mice.

Protocols were approved by the University of Guelph's Animal Care Committee. 30 C57 females (from Charles River, Quebec) were housed from 4 weeks of age in mixed strain triplets (for the purposes of another study: two C57s plus one DBA/2; see [17] for validation), in 15 standard woodchip-bedded laboratory cages (12cm H X 27L x 16W), each provided with Shepherd Enviro-dri© nesting material and a UDEL polysulfone plastic mouse house shelter, plus *ad libitum* food (Harlan® Teklad Global Diet) and water. One C57 per cage was ear-notched for identification. Cages were maintained at 21°C, on a 12:12 reversed dark/light cycle (lights off at 1000h). After five months, behavioural observations were conducted. Mice were live scan-sampled *in situ*, using red room lights/headlamps, to assess activity budgets. Scans were taken every 20 minutes, for four hours per session (1130-1530h or 1730-2130h), repeated over four days (thus eight sessions): a method based on [17]. Scans were split between two experimenters (LH and KR; inter-observer reliability: >95%). Table 1 provides the ethogram (modified from [18]). SB was calculated as a percentage of both overall activity and observations. However, these covaried tightly ($F_{1,\ 15.2} = 914.38$, $P < 0.0001$, $R^2 > 0.99$) and gave near identical results, so only results for the former are presented.

Subjects were killed by cervical dislocation when 7 months old. Brains were extracted immediately and drop-fixed in cold 4% paraformaldehyde, PFA (cf. e.g. [19]), the PFA being refreshed twice within the first 24h to aid fixation. After c. 4 weeks in PFA (stored at 4 degrees), brains were then transferred into cryoprotective 30% sucrose in phosphate buffered saline (PBS) for 48 hours, and tissue was then sliced using a Leica

Cryostat into 30μm coronal sections collected in series, mounted onto gelled slides, and stored at -80˚C until staining.

In six of the cages, both C57s were clearly stereotypic (spending 3.5-55.5% of their active time budget in SB). In the remaining nine cages, they performed negligible SB (0-1.5% of the active time budget). Data from cagemates are not statistically independent [20], and correspondingly, the SB levels of the two C57s per cage tightly covaried ($F_{1, 13} = 111.89$, $P < 0.0001$). The brain of one mouse was therefore chosen from each of the six stereotypic cages, and one subject randomly chosen from five of the others (by GM, to ensure the researcher performing staining and quantification [DP] was blind). One slide was chosen per mouse, from which two adjacent sections were selected (using landmarks [cf. 14, 21] ensuring staining of both caudate-putamen and nucleus accumbens).

To develop the FosB/ΔFosB immunohistochemical procedure (the antibody used [Sc-48, Santa Cruz Biotechnology, CA] was anti-ΔFosB but cross-reacted with FosB), protocols were obtained from authors of relevant studies [14, 22, 23, 24]. These varied, so were combined and optimized via pilots on spare tissue. In the final protocol, all conducted at room temperature, slides were post-fixed in 4% PFA for 10 minutes, rinsed in PBS, then exposed to H_2O_2 (1% in PBS) for 10 minutes. The samples were blocked (PBS, 1.5% Triton X and 3% goat serum) for 1 hour, incubated in primary antibody (diluted 1:500 in PBS with 0.3% Triton X and 0.03% sodium azide) for 22 hours, then incubated in biotinylated goat anti-rabbit secondary antibody (1:200, Vector Laboratories, Burlingame, CA) for 2 hours. The amplification step used an avidin/biotin peroxidase complex (Vectastain ABC Kit, Vector Laboratories) for 90 minutes. Immunoreactivity was revealed by incubating slides for 12 minutes in 0.06% DAB and 0.1% H_2O_2, diluted in PBS. Sections then were rinsed in PBS, dehydrated in increasing concentrations of ethanol (50% for 1 minute, 70% for 1 minute, 95% for 3 minutes, 100% for 3 minutes), dipped in xylene for 2 hours, then coverslipped using DPX Mountant (Fisher Scientific). Additional control slides underwent each step except the primary antibody, to ensure background staining was absent.

Images were taken using the x10 objective of a Leica DMR HC Brightfield microscope, and a sample of 200x200 pixels selected from each of five regions: the NAc,

and four CPu areas (dorsal, ventral, medial and lateral; cf. e.g. [6, 14, 21]; see Figure 1). These images were taken bilaterally from the two consecutive sections, resulting in four samples per region per mouse. Stained nuclei were identified by dark nuclear coloration surrounded by lighter stained cytoplasm (Figure 2), published images of successful FosB/ΔFosB immunolabeling being used as guides [23]. Dead cells were also counted (because these could act as confounds by preventing positive staining), easily identified via nuclear condensation (see Figure 2). Both were counted manually.

Because of weak staining compared to published studies [e.g. 14, 23] and concerns over cell deaths, we first ran extensive checks for data quality and consistency of staining. This included assessing the scorer (DP)'s internal consistency, a subset of 25 regions being randomly selected for a blind re-count of cells positively stained for FosB/ΔFosB and dead cells. Here, as throughout, data were analysed using General Linear Models (GLMs) in JMP 12.0, and Box-Cox transformed when needed to meet the assumptions of parametric statistics (Spearman's tests being used when this was unsuccessful). First counts strongly predicted blind re-counts ($P < 0.0001$ for both cell types) indicating high intra-rater reliability. Next, relationships between positively stained and dead cell counts between the two consecutive sections per hemisphere were assessed. Both positive counts and dead cells counts from successive sections positively correlated ($P < 0.05$ in all tests), save for the right hemisphere's NAc ($P > 0.05$ in both tests). Because successive sections were thus generally similar, values from each pair were averaged for subsequent analyses. A series of GLMs then assessed whether positive counts for each region covaried between the two hemispheres. Dead cell counts were statistically controlled for by being added as covariates (since they compromised staining, samples with high dead counts having low positive counts: $P < 0.05$ in all analyses; also see Fig. 4a). Positive counts significantly covaried between hemispheres for the NAc, and dorsal and lateral CPu regions, and showed a strong similar trend in the medial CPu ($P < 0.054$ in all tests). Because overall, staining levels thus typically covaried between hemispheres, right and left hemisphere values were averaged for subsequent analyses. Finally, inter-relationships between positive counts in all four CPu sub-regions were investigated for staining consistency, again statistically controlling for dead cells. All

significantly covaried ($P < 0.05$ in all tests), and so were averaged for subsequent analyses. By contrast, none covaried with NAc counts ($P > 0.05$ in all analyses).

Investigating the relationships between SB and basal ganglial FosB/ΔFosB staining revealed no significant relationship between SB and positively stained cell counts within the CPu ($F_{1,8} = 0.53$, $p=0.49$). To explore further, SB was regressed against the positive counts for each individual CPu region, but again, no relationships were found ($P > 0.05$ in all tests). However, there was a positive correlation with SB in the NAc ($F_{1,8} = 9.27$, $p=0.016$). This GLM again statistically controlled for dead cell counts, and also used Box-Cox transformed data, making this significant relationship hard to convey in a figure. Raw data were therefore plotted and analysed with a Spearman's test (see Figure 3). The analysis was also re-run in a two-step manner conceptually similar to the GLM but, again, easier to present graphically (see Figures 4a and 4b).

Our findings thus implicate the ventral striatum in the cage-induced SB of C57 laboratory mice, consistent with Cabib's hypothesis [3, 10] and suggesting an aetiology similar to that of 'hyper-motivated' compulsive gambling, drug-taking and eating, and stimulant-induced hyper-activity [1, 2, 5, 25]. Our primary interest was to identify structures playing a causal role in SB. Elevated striatal (especially NAc) ΔFosB does influence behaviour, for instance promoting reward-seeking, wheel-running and impulsivity in food-rewarded tasks (e.g. [16, 25]). We may therefore have successfully identified a cause of SB. However, we cannot *assume* this type of causality from mere correlation. Indeed, caution is urged by the way that NAc FosB elevates in response to motivationally salient stimuli, both stressors and rewards [14-16, 25]): findings that suggest two alternative explanations for our results. One is that NAc ΔFosB and SB are independently increased by stress, with no causal connection between them; thus high SB mice find their cages particularly stressful (which elevates ΔFosB), and are highly motivated to escape (escape-attempts developing into SB: [4]). Alternatively, performing SB could be rewarding, such that its performance *causes* increased NAc FosB. Careful experimental work is now needed to distinguish these hypotheses.

Our findings further suggest that the aetiology of this SB diverges from classic amphetamine stereotypies or other repetitive behaviours induced by motor loop over-activation [1, 5, 7]. Completely discounting involvement of the dorsal striatum is

premature given our small sample size, but, if present, its effects are clearly relatively weak. This conflicts with suggestions that C57 SB reflects motor loop dysfunction [5]. One possible explanation is that response repetition in gambling tasks [5] is simply not a good diagnostic of this, instead being sensitive to NAc activity. However, it is also possible that the two sub-populations of C57s studied differed in the neurological bases of their SB. For example, the Garner work [5] used mice that, at 5-15 months of age, were largely older than ours; furthermore both our studies pooled several types of SB (e.g. jumping, route-tracing and bar-mouthing) that could have occurred in differing ratios: important if these diverse forms have heterogeneous aetiologies. In the future we therefore plan to replicate this promising use of FosB/ΔFosB immunohistochemistry to understand SB, combining it with finer distinctions between different SB forms, along with measures of both response repetition in gambling tasks (cf. [5]) and impulsive responses to rewards (cf. [1, 10, 25]).

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227 **Table 1: Behaviour recorded during scan-sampling**

Category	Description
Stereotypic behaviour	Bar mouthing: mouse holds cage bar in mouth for 1s or longer while making movements along it
	Route tracing: mouse runs over cage floor in a pattern for three or more repetitions
	Patterned climbing: climbing on cage lid in a pattern for three or more repetitions
Other Activity	Moving, digging, carrying/manipulating nest materials; moving across cage in a non-stereotypic way; grooming; movement in nest/shelter although out of clear sight of experimenter; feeding; drinking; chasing or being chased; displacing another mouse from the feeder, or being displaced.
Inactive	Mouse is still (this includes eyes closed presumed sleeping; eyes open; and out of sight but not moving)

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FIGURE LEGENDS

Figure 1: Diagram of coronal section of mouse brain (at bregma + 1.10, modified from [14]), showing where dorsal CPu (1), medial CPu (2), ventral CPu (3), lateral CPu (4) and NAc (5) images were sampled (the latter not aiming to distinguish core from shell, but instead to approximately span both regions). 'lv' = lateral ventricle; 'ac' = anterior commissure.

Figure 2: One typical image (see text for details), here of medial caudate-putamen, highlighting four examples of FosB/ΔFosB stained nuclei (filled arrows) and two examples of dead cells (unfilled arrows). NEEDS SCALE

Figure 3: Counts of cells in the NAc that positively stained for FosB/ΔFosB, plotted against SB level (Spearman rho = 0.67, $P < 0.05$). For how this positive relationship improves if dead cell counts are statistically controlled for, please see text and Figures 4a and b.

Figure 4a: Counts of cells that were positively stained for FosB/ΔFosB, or dead, in the NAc of each mouse, along with the line of best fit (dashed) for this negative relationship. The values by each datapoint show that mouse's SB level. The "residual" measure for positive counts is the vertical distance between each datapoint and the line (e.g. as shown by the vertical dotted line for the subject with 18.5% SB). Positive residuals thus mean high positive counts of FosB/ΔFosB stained cells for the number of dead cells in any given sample, while negative residuals mean low positive counts for the number of dead cells. By inspection, the more stereotypic mice have more positive residuals (tested statistically in Fig. 4b).

Figure 4b: Residual values for positive counts of FosB/ΔFosB stained cells in the NAc (positive residuals – those to the right of the dashed line – meaning many positive counts

for the number of dead cells in a given sample), plotted against each animal's SB. The asterisk indicates the 18.5% SB mouse highlighted in Fig. 4a. Mice with higher residual values are more stereotypic (Spearman rho = 0.77, $P < 0.01$): an effect stronger than that in Fig. 3 because of this correction for dead cell counts (cf. the GLM reported in the text).

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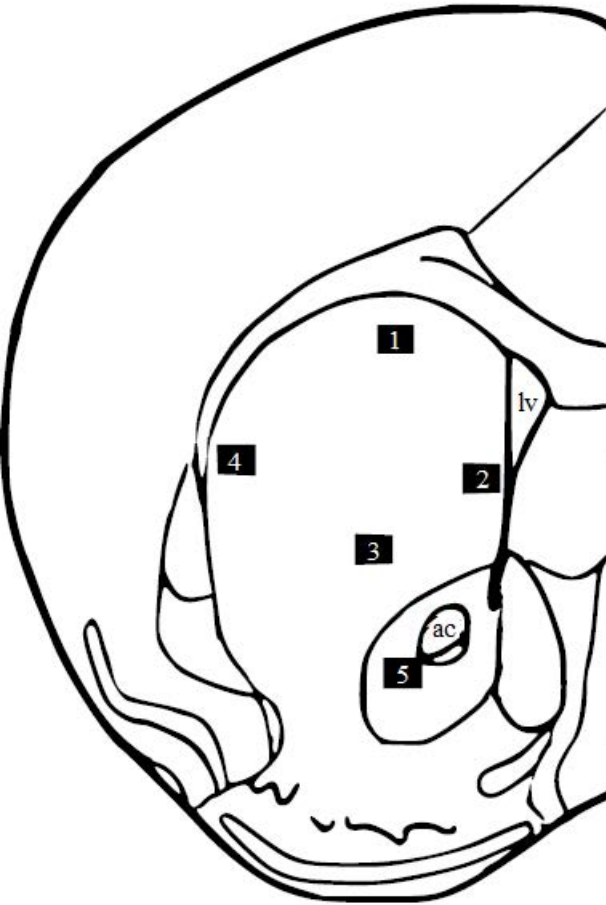

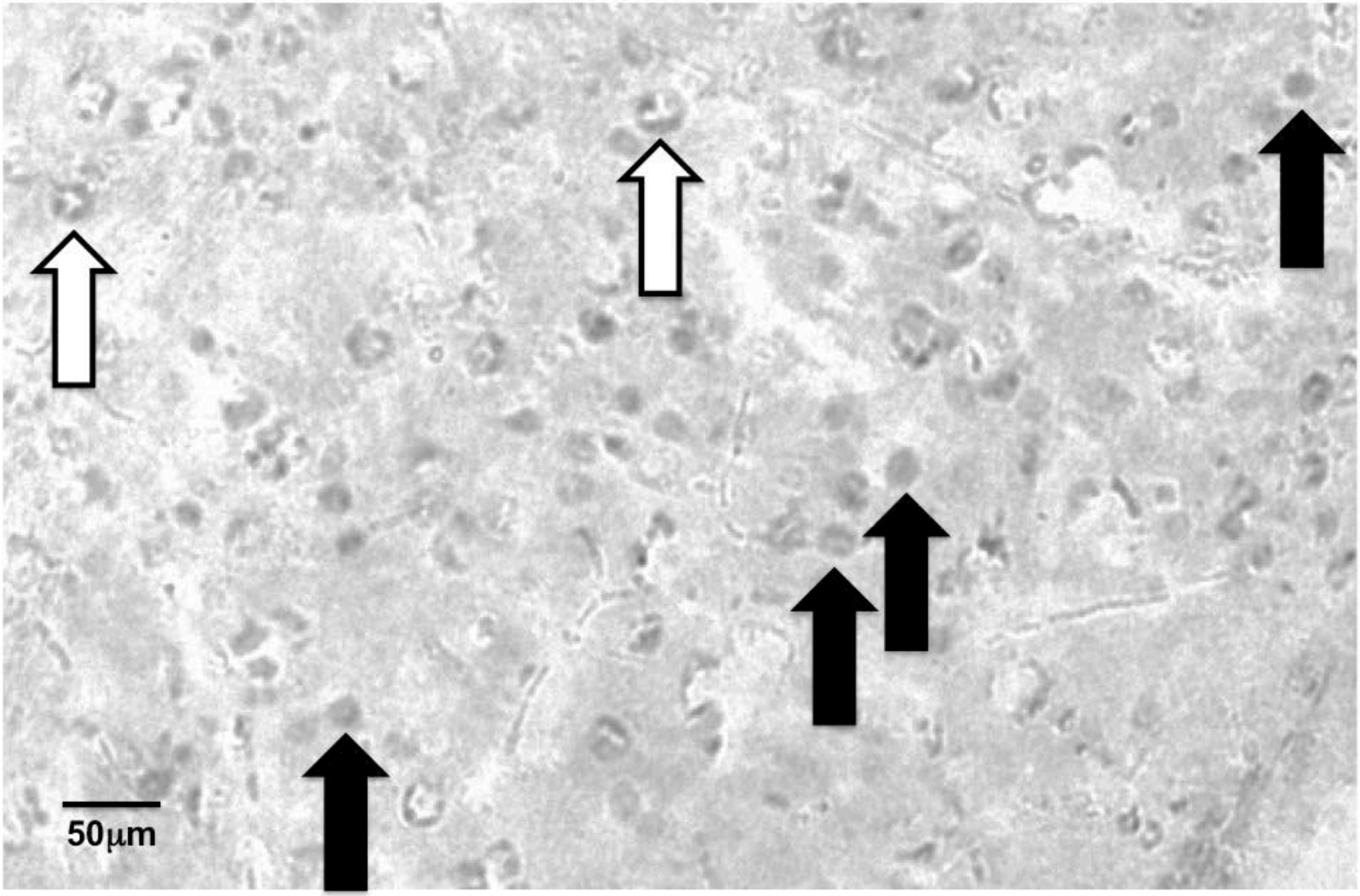
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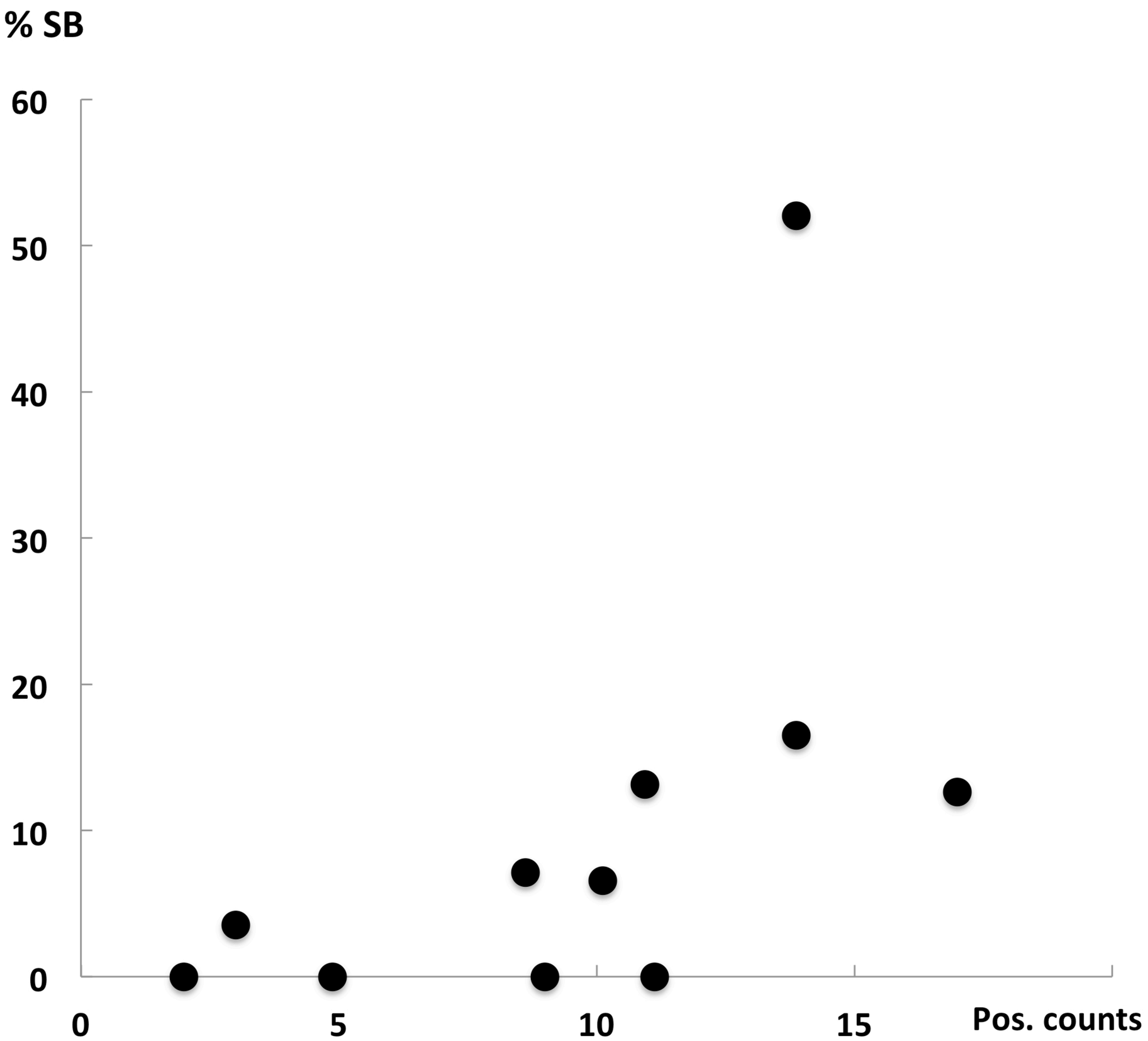
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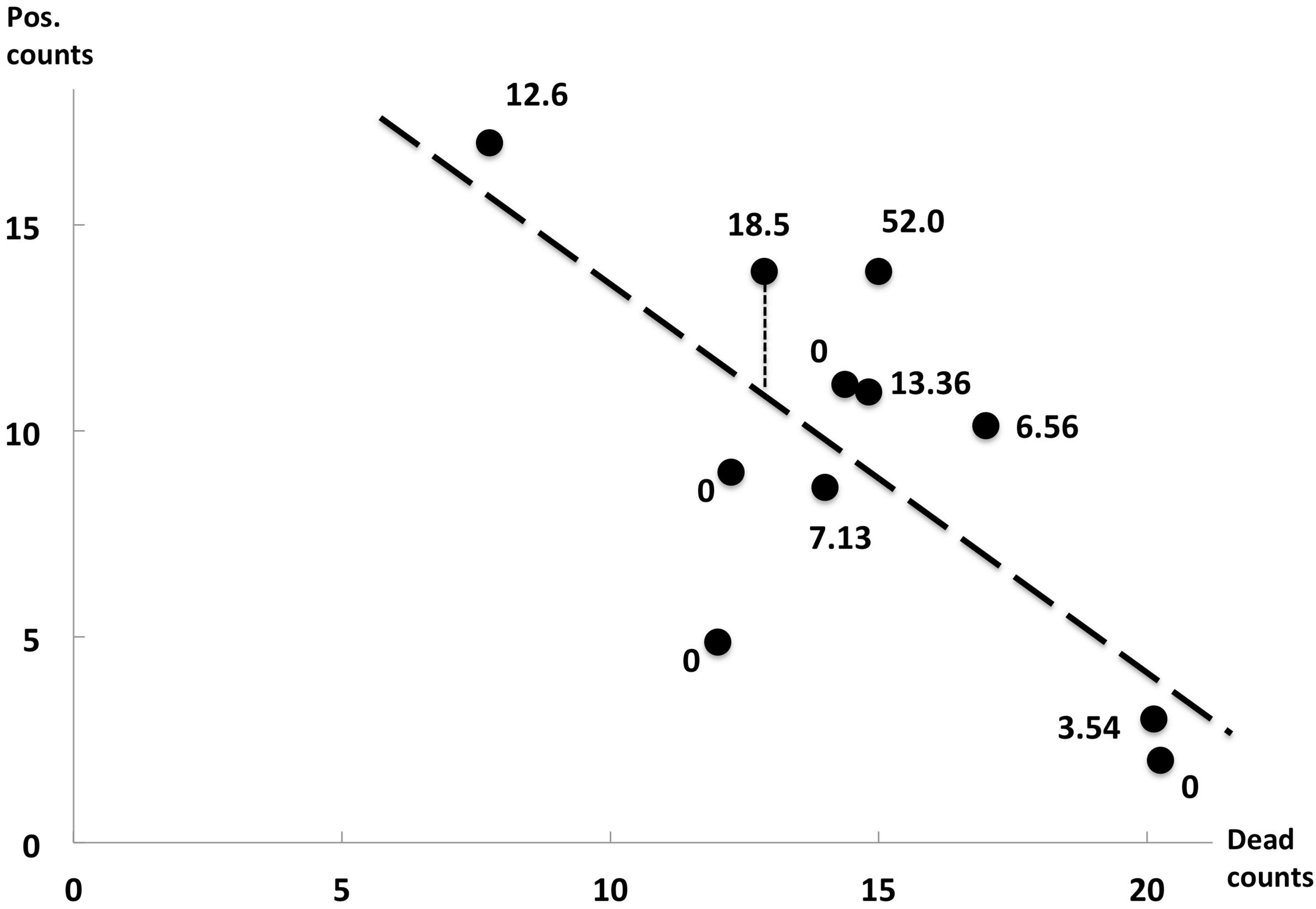
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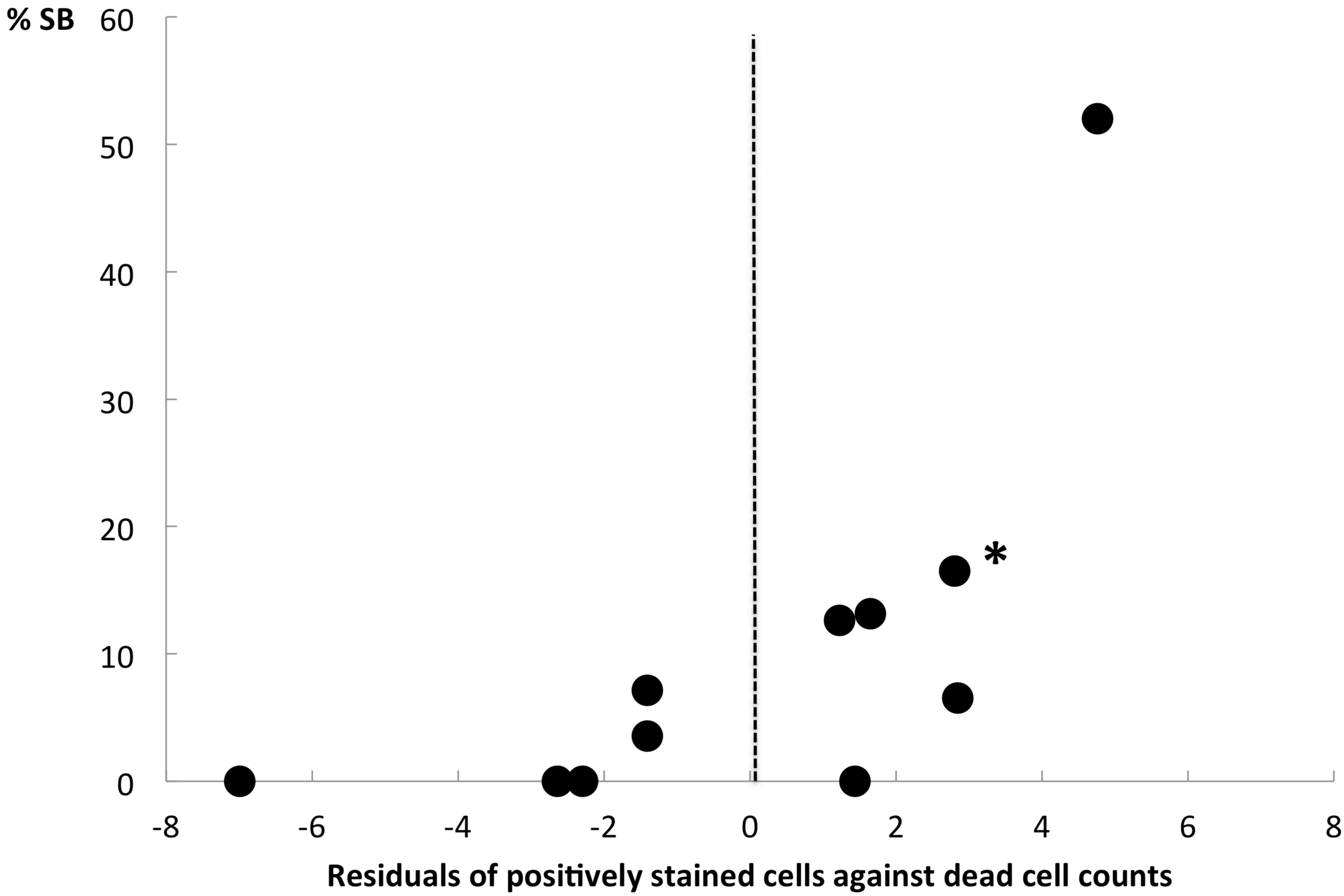
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