Effects of different blood collection methods on indicators of welfare in mice

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Blood collection is a common experimental procedure for which there are many different methods, each with its own advantages and disadvantages. Researchers should use methods that minimize pain, suffering, distress and lasting harm to animals while meeting study requirements. The authors evaluated stress, activity and tissue damage in BALB/c01aHsd mice after collecting blood using one of six methods: retrobulbar bleeding with thin or thick capillaries, tail vein bleeding, saphenous vein bleeding, facial vein bleeding or jugular vein bleeding. The authors compared in-cage activity, corticosterone concentration and performance in open-field tests between treatment groups and collected histologic samples at 1 h, 3 d and 14 d after bleeding. Mice that underwent retrobulbar bleeding with a thick capillary had a smaller change in corticosterone concentration and higher in-cage activity immediately after blood collection, whereas mice that underwent jugular vein bleeding had a greater change in corticosterone concentration and lower in-cage activity and open-field activity. Mice that underwent saphenous vein bleeding had a high incidence of histological change at 1 h, 3 d and 14 d after blood collection, but few indicators of histological change were present in other groups at 14 d after blood collection. These results suggest that, when collecting a small volume of blood, retrobulbar bleeding with a thick capillary and without anesthesia causes the least stress in mice, whereas jugular vein bleeding and facial vein bleeding cause the most stress and saphenous vein bleeding causes the most lasting damage in mice.

Various methods can be used to collect blood samples from rats and mice. When selecting which method to use, researchers must consider many factors, including the welfare of the subject animals. It is a researcher's responsibility to select an effective method that minimizes pain, suffering, distress and lasting harm[1]. Research and institutional guidelines recommend the use of several blood collection methods with rats and mice[2–4].

In retrobulbar bleeding (RBB), also known as retro-orbital bleeding, a capillary tube is used to disrupt the retrobulbar venous sinus located behind the eye. This technique can be used to collect a large volume of blood with or without anesthesia, typically without detriment to the subject animal's general health. It is widely used for bleeding or exsanguinating rats and mice[5–7] and meets animal welfare guidelines[2], but its effects on stress and animal welfare depend on the speed and ability of the technician. An experienced technician can avoid severe tissue damage[8], but RBB remains controversial owing to the risk of damaging the Harderian gland and other tissues surrounding the eyes[9,10].

In tail vein bleeding (TVB), a needle is used to puncture one of the veins located in the tail. It is a simple technique for collecting small volumes of

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blood from unanesthetized mice or rats[11,12], but this procedure requires that animals be warmed to dilate the vein before bleeding[13]. It can also take much more time to collect a given volume of blood by TVB than by RBB or SVB[14,15] owing to the low blood pressure and flow rate of the tail. This prolongs handling of the rodent, which can cause stress; consequently, corticosterone concentrations can be higher in blood samples collected by TVB than in those collected by RBB[16].

In saphenous vein bleeding (SVB), a needle is used to puncture the saphenous vein at a prepared site near the ankle. It is a fast technique for serial blood collections from unanesthetized mice or rats, but it requires that the puncture site be shaved first. The superficial location of the vein facilitates accurate venipuncture and observation of any post-collection hemorrhage[17]. Persistent (minor) bleeding is the only reported complication for this technique[3]. Anesthesia is not required but conscious animals experience some stress associated with restraint, whether by hand or by restraint tube. Multiple studies have examined how SVB affects behavior, body weight and plasma concentrations of corticosterone and glucose in mice[13,15,18,19].

In facial vein bleeding (FVB), also known as submandibular bleeding, a lancet is used to puncture the facial vein located near the back of the jaw. This is a commonly used, fast technique for blood collection from unanesthetized mice or rats. If carried out improperly, this technique can penetrate and damage the inner ear or lacerate facial muscles and nerves[20,21], although commercially developed lancets are designed to reduce these risks by limiting the depth of puncture below the skin[22]. A few studies have found this technique to be more stressful than RBB[6,23], TVB[13,21] and SVB[19].

In jugular vein bleeding (JVB), a needle mounted on a syringe is used to puncture the jugular vein in the craniocervical region. This is an effective technique for repeated bleeding of small animals and may cause fewer negative effects than RBB or TVB in mice or rats[5,16,24,25]. When used for serial blood collections from unanesthetized mice, it causes stress responses similar to those caused by the tail incision technique, in which a razor blade is used to incise the lateral tail vein[26]. JVB is recommended by Gesellschaft für Versuchstierkunde/Society of Laboratory Animal Science[2] only when carried out by well-trained persons on anesthetized animals. There is little detailed information about the effects of this technique in mice and rats.

Some research has examined the effects of different blood collection techniques on physiology and behavior in rats[5,7–9,12,27–30], but most evaluations with mice have focused on blood quality or refinement of a method, especially for repeated bleeding[31–33]. None has carried out a comprehensive evaluation of the effects of different blood collection techniques on the welfare of mice. This study compares the physiological, behavioral and histopathological effects of recommended bleeding techniques[2] in mice.

Corticosterone is the principal glucocorticoid involved in regulation of fuel metabolism, immune reactions and stress responses[13]. It is therefore a useful parameter for measuring acute stress, and, in mice, corticosterone levels continue to increase for as long as 15 min after exposure to a stressor[34,35]. In-cage activity can be used as a parameter for assessing the influence of experimental treatment, genetic mutations or drug effects on rodent behavior[36]. The open-field test is also a common measure of exploratory behavior and general activity in mice and rats[37]. Stress can decrease locomotion and exploration activity and increase anxiety in mice[38]. Reserpine-induced pain also can decrease the total travel distance of mice during open-field tests[39]. We quantified changes in in-cage activity, corticosterone concentration and open-field test performance in mice to evaluate the stress response associated with each method of blood collection.

METHODS

Mice and husbandry

We purchased 72 6-week-old, female, specific pathogen–free[40] BALB/cO1aHsd mice from Harlan Laboratories (Borchen, Germany) for this study. The mice were marked by ear puncturing, randomly allotted to six experimental groups (n = 12) and housed in groups of six in Makrolon type III cages (Tecniplast, Buguggiate, Italy), such that each cage housed one mouse from each experimental group. Mice were given *ad libitum* access to tap water in drinking bottles and pelleted food containing 19.0% protein, 4.0% fat, 6% fiber and 7% ash (No. 1324, Altromin GmbH, Lage, Germany). Soft wood shavings were provided in each cage as bedding (Type 5, Altromin GmbH, Lage, Germany). Cages and bedding were changed every 7 d and nest material (Nestlets, EBECO, Castrop-Rauxel, Germany) was provided in each cage after changing as an environmental improvement. Cages were kept inside a Scantainer ventilated cabinet (Scanbur AS, Lellingegaard, Denmark) with the air exchanged 10–16 times per hour and a light intensity of 50 ± 10 lux on a 12-h:12-h light:dark cycle (with the lights coming on at 6:00 a.m.). Room temperature was 22 ± 2 °C with 55 ± 10% relative humidity. All husbandry and experimental procedures were carried out in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes and the Animal Welfare Act of Germany.

Mice were handled daily between 9:00 a.m. and 12:00 p.m. during a 14-d acclimatization period before treatment to reduce later handling stress during the experiment. During handling each animal was placed on a handler's arm for at least 1 min.

Pretreatment blood collection

Five days after the mice arrived at the facility, we began collecting blood samples to measure pretreatment corticosterone concentration in each mouse. We collected blood from a different mouse from each cage every day for 6 d, which minimized and standardized the stress associated with opening the cage. We carried out these blood collections between 9:00 a.m. and 12:00 p.m. each day and collected 150 µl blood from each mouse into a 0.5-ml centrifuge tube (Eppendorf, Hamburg, Germany). We collected blood by RBB from the left eye without anesthesia, using two thick capillaries, each with an outer diameter of 1.5–1.6 mm (volume of 75 µl, Hirschmann, Eberstadt, Germany). This type of capillary was used in all blood collections during this study, except RBB_B and JVB treatments. We centrifuged the blood samples at 12,000 r.p.m. for 5 min and extracted the blood plasma. Plasma was stored in a freezer at −20 °C until all blood samples were collected and was then thawed for corticosterone analysis.

Blood collection treatment

At the time of treatment, the mice were 8 weeks old and weighed 19.26 ± 1.21 g (mean ± s.d.). We collected blood from the mice in each of the six groups using a different bleeding method: one of two different RBB techniques, TVB, SVB, FVB or JVB. We collected blood from a different mouse from each cage every day for 6 d between 9:00 a.m. and 12:00 p.m. For each blood collection treatment, the subject mouse was removed from its housing cage and transported to the adjacent experimental room in a Makrolon type II cage (Tecniplast, Buguggiate, Italy) with bedding. A technician began the blood collection procedure immediately after the mouse arrived in the experimental room. We recorded the amount of time spent on the entire treatment procedure, beginning with the mouse's removal from its home cage and ending when the mouse was awake in the transportation cage after blood collection. We also recorded the amount of time spent on just the bleeding technique, beginning with the mouse's restraint and immobilization and ending when 75 µl blood was collected.

All blood collection techniques were carried out by experienced technicians or researchers, who routinely apply the technique in their respective institutions, to minimize possible tissue damage[8] or stress in mice[18]. Five technicians or researchers traveled from different institutions in Germany to the Institute for Animal Welfare and Behavior of the University of Veterinary Medicine Hannover to participate in this study. Each bleeding technique was carried out by a single technician every time. We prepared and arranged blood collection equipment for all technicians such that working conditions were the same in this study as at their home institutions.

When anesthetizing a mouse, the technician placed the mouse in a box measuring 15 cm × 13 cm × 28 cm and induced narcosis by introducing 8 ml ether vaporized with 5 l/min oxygen[41] inside a gas-washing bottle. This setup was specifically designed to introduce gaseous anesthetic agents and included an extra exhaust outlet for human safety. An anesthetist attended this procedure and noted when the mouse's respiration became visibly depressed, whereupon the technician removed the mouse from the box. For JVB, each mouse was kept under inhalation anesthesia for the entire bleeding procedure. To maintain inhalation anesthesia, the technician placed the mouse's nose in a 5-ml tube containing two cotton balls soaked in 0.5 ml ether (**Fig. 1**). Once removed from anesthetic agents, mice were determined to have emerged from anesthesia once they had righted themselves from a supine position.

RBB. We compared two different RBB techniques. In each, the technician manually restrained each mouse at the neck and inserted a hematocrit capillary into the medial canthus of the right eye (**Fig. 2**). The technician then rotated the capillary along the bulbus of the eye, rupturing the retrobulbar venous sinus (*sinus orbitalis*). For RBB_A the technician used a thick capillary with an outer diameter of 1.5–1.6 mm (volume of 75 µl; Hirschmann, Eberstadt, Germany) to collect blood without anesthetizing the mouse. The thick capillary remained in the eye until it collected 75 µl blood. For RBB_B the technician induced anesthesia in the mouse and used a thin capillary with an outer diameter of 1.2 mm (volume of 20 µl; intraEND, Blaubrand, Wertheim, Germany). Once inserted, the thin capillary remained in the eye until it collected 75 µl blood into a 0.5-ml centrifuge tube (Eppendorf, Hamburg, Germany). The technician then removed the capillary and stopped bleeding with a cotton pad.

TVB. For TVB the technician first warmed each mouse in its transportation cage with a red light

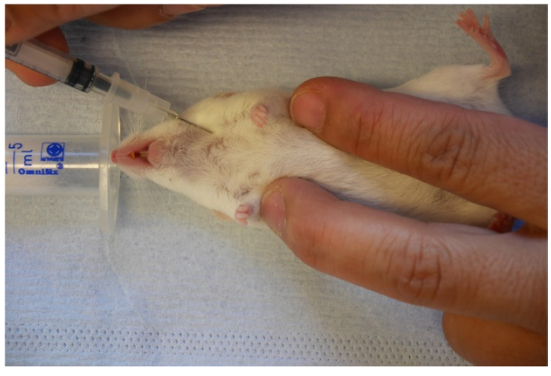

FIGURE 1 | Jugular vein bleeding. The technician punctures the venous angle under the clavicle with a needle mounted on a syringe and draws blood into the syringe.

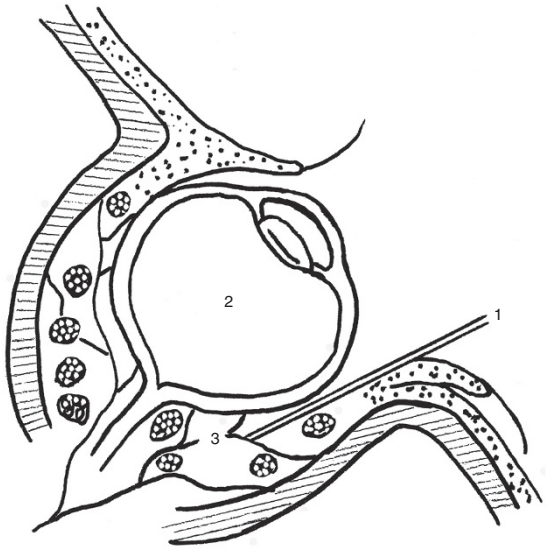

FIGURE 2 | Retrobulbar bleeding. The technician rotates the capillary (1) along the bulb (2), disrupts the retrobulbar venous sinus (3) and draws blood into the capillary. Image is drawn based on ref. 45.

(250 W, 230–250 V; Albert Kerbl, Buchbach, Germany). After 10 min, the technician restrained the mouse in a custom-made restraint and used a 23-gauge needle (B. Braun, Melsungen, Germany) to puncture the right lateral tail vein (*v. caudalis lateralis*). The technician then collected 75 µl blood using a thick capillary (**Fig. 3**) and stopped bleeding with a cotton pad.

SVB. For SVB the technician restrained each mouse in a 50-ml tube (BD Biosciences, Heidelberg, Germany). The technician isolated the leg above the knee and shaved away fur with a GT420 electric shaver (Braun, Suhl, Germany) until the saphenous vein was visible and then used a 27-gauge needle (B. Braun, Melsungen, Germany) to puncture the lateral saphenous vein (*v. saphena lateralis*). The technician then collected 75 µl blood using a thick capillary (**Fig. 4**) and stopped bleeding with a cotton pad.

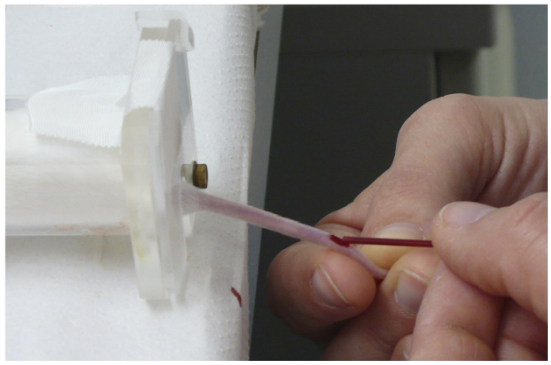

FIGURE 3 | Tail vein bleeding. The technician punctures the right lateral tail vein with a needle and draws blood into a capillary.

FIGURE 4 | Saphenous vein bleeding. The technician punctures the lateral saphenous vein with a needle at a shaved location above the knee and draws blood into a capillary.

FVB. For FVB the technician manually restrained each mouse and punctured the facial vein (*v. facialis*) using an Accu-Check lancet (Roche, Mannheim, Germany; **Fig. 5**). The technician then collected 75 µl blood using a thick capillary and stopped bleeding with a cotton pad.

JVB. For JVB the technician first induced anesthesia in each mouse, then maintained anesthesia and placed the mouse on its back with its legs stretched caudally. The technician mounted a 27-gauge heparinized needle on a 1-ml syringe (B. Braun, Melsungen, Germany) and inserted the needle into the craniocervical region under the clavicle towards the opposite knee to puncture the venous angle (*angulus venosus*), as previously described in literature[16] (**Fig. 1**). The technician aspirated the syringe until it contained 75 µl blood, then withdrew the needle and stopped bleeding with a cotton pad.

In-cage activity

Immediately after the blood collection treatment, we placed each mouse in the transportation cage and recorded its in-cage activity for 10 min using a

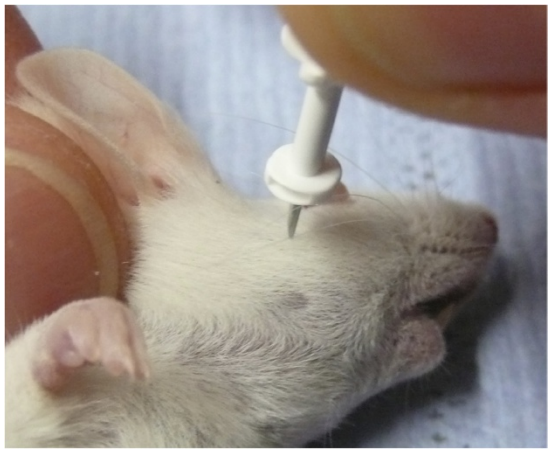

FIGURE 5 | Facial vein bleeding. The technician punctures the facial vein with a lancet and draws blood into a capillary.

TABLE 1 | Scoring for in-cage activity

Scoring	Characteristics
1	Immobile
2	Minimal movement
3	Slow movement
4	Visible interest in the environment
5	Visible interest and unsteady movements
6	Exploratory/locomotor behavior, not completely steady
7	Active with strong exploratory/locomotor behavior

Canon G10Hi camera with Hi8 tapes (Canon, Tokyo, Japan). We began recording immediately upon placing a mouse in the cage, unless the mouse had undergone RBB_B or JVB, in which case we began recording activity upon emergence from anesthesia. We used a different transportation cage for each mouse and cleaned these cages between days. After the experiment, we reviewed each recording of in-cage activity, rating the activity level of each mouse on a scale of 1–7 and recording the amount of time spent by each mouse at each activity level. We then calculated the average activity level for the mouse over the entire 10-min recording (**Table 1**).

Post-treatment blood collection

Fifteen minutes after the blood collection treatment was complete, we collected 150 µl blood from the left eye of each mouse into a 0.5-ml centrifuge tube (Eppendorf, Hamburg, Germany) by RBB without anesthesia using two 75-µl capillaries. We centrifuged the blood samples at 12,000 r.p.m. for 5 min and extracted the blood plasma. Plasma was stored in a freezer at −20 °C until all blood samples were collected and was then thawed for corticosterone analysis.

Corticosterone measurement

We carried out a competitive corticosterone ELISA (IBL, Hamburg, Germany) and measured plasma corticosterone concentrations using a microtiter reader at 450 nm. For each mouse, we calculated the change in corticosterone concentration by subtracting the pre-treatment corticosterone concentration from the post-treatment corticosterone concentration.

Open-field test

Immediately after collecting blood for corticosterone analysis, we placed the subject mouse into the center of an open-field apparatus similar to one previously described[42], measuring 60 cm × 60 cm square with a wall height of 50 cm. We recorded the behavior of the mouse for 5 min using a Model ICD-47E camera (Ikegami, Tokyo, Japan) with a TL 550 video recorder (Panasonic, Osaka, Japan). We analyzed each recording after the experiment using EthoVision 3.1 software (Noldus, Wageningen, the Netherlands) and calculated the latency of the mouse to move toward the edge of the apparatus, the total distance traveled by the mouse and the average speed of the mouse. After open-field testing, we returned the subject mouse to its home cage and cleaned the open-field apparatus.

Histological examination

We collected histopathological samples of puncture sites 1 h, 3 d and 14 d after the blood collection treatment. We euthanized four mice from each experimental group at each time point ($n = 4$) by carbon dioxide inhalation, according to a previously described method[43]. Carbon dioxide was introduced into home cages through a customized lid to produce a homogenous distribution and at a rate of 13 l/min until breathing ceased. After euthanasia, we isolated the puncture site for each mouse, fixed tissues with 10% formaldehyde solution and prepared histologic slides. We embedded tissues in paraffin, cut three sections of 3–4 µm at 100-µm intervals using a rotary microtome (Mikrotom HM 360, Microm, Walldorf, Germany) and stained these sections with hematoxylin and eosin.

We examined these sections under a microscope and noted the presence of eight indicators of histological change (hemorrhage, blood clots, polymorphonuclear inflammatory cells, mononuclear inflammatory cells, degenerative or necrotic changes, fibrosis, edema and thrombus) in four types of tissue (skin tissue, blood vessels, muscle tissue and connective and adipose tissue). For mice that underwent RBB, we evaluated two additional tissue types: Harderian gland and optic nerve. We assigned one point for the presence of each indicator in each tissue type in each mouse and calculated the incidence of histological change in each group as a percentage of the total possible score for that group, which was 128 points for groups that underwent TVB, SVB, FVB and JVB and 192 points for groups that underwent RBB.

Data analysis

We analyzed all data using StatView software version 5.0 (SAS Institute Inc., Cary, NC). We used the Lilliefors test to check all data for normality and confirmed that all data were normally distributed, including average in-cage activity scores. We carried out one-way analysis of variance tests (ANOVAs) to compare bleeding methods with respect to the following variables: time spent on the entire treatment procedure, time spent on just the bleeding technique, in-cage activity level of mice, change in corticosterone concentrations in mice, latency of mice to move to the edge of the field in the open-field test, total distance traveled by mice in the open-field test and average speed of mice in the

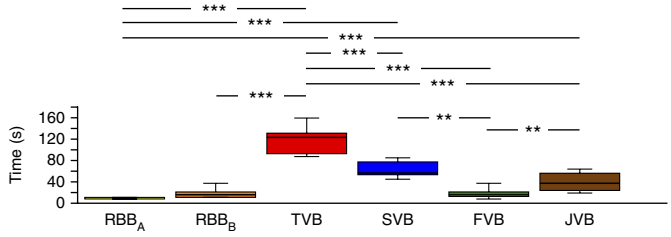

FIGURE 6 | Length of time spent on just the bleeding technique was significantly shorter for RBB_A and FVB than for SVB and JVB and was significantly longer for TVB than for any other technique. Boxes indicate interquartile ranges, lines inside the boxes indicate medians and whiskers indicate the sample range (minimum–maximum). $^{**}P < 0.05$; $^{***}P < 0.001$.

open-field test. For these analyses we carried out post-hoc pairwise comparisons using Scheffé tests. We carried out paired *t*-tests to compare plasma corticosterone concentrations in pre-treatment samples and post-treatment samples for each treatment group. For all analyses $P < 0.05$ was considered statistically significant and data are expressed as mean ± s.d.

RESULTS

Blood collection

The length of time spent on the entire treatment procedure differed significantly between treatments ($F_{5,66} = 99.326$, $P < 0.0001$). This length of time was shortest for RBB_A (9.42 ± 1.24 s) followed by FVB (20.50 ± 11.80 s), SVB (63.17 ± 16.12 s), RBB_B (118.50 ± 24.33 s), JVB (136.75 ± 21.64 s) and finally TVB (716.91 ± 25.38 s). The length of time spent on just the bleeding technique also differed significantly between treatments ($F_{5,66} = 29.597$, $P < 0.0001$). This length of time was significantly shorter for RBB_A (6.43 ± 1.24 s) and FVB (13.33 ± 11.33 s) compared with SVB (40.67 ± 13.82 s) and JVB (40.58 ± 16.58 s; $P < 0.05$ for these comparisons). The length of time spent on just the bleeding technique of RBB_B (18.67 ± 9.89 s) was also generally shorter than SVB ($P = 0.0727$) and JVB ($P = 0.0747$). TVB took significantly longer (79.09 ± 31.52 s) than all other techniques ($P < 0.001$ for these comparisons; **Fig. 6**).

In-cage activity

Average in-cage activity level differed significantly between treatment groups ($F_{5,66} = 65.752$, $P < 0.0001$). Activity level was significantly lower in mice that underwent FVB and JVB than in mice that underwent RBB_A, SVB or TVB ($P < 0.0001$ for these comparisons). Activity level in mice that underwent RBB_B was also significantly lower than that in mice that underwent RBB_A or TVB ($P < 0.05$ for both comparisons) but significantly higher than that in mice that underwent FVB or JVB ($P < 0.0001$ for both comparisons; **Fig. 7**).

Corticosterone concentration

Plasma corticosterone concentration was significantly higher after treatment than before treatment in each treatment group ($P < 0.05$ for all comparisons; **Fig. 8**). The increase in corticosterone concentration after treatment differed significantly between groups ($F_{5,66} = 2.523$, $P = 0.0382$). This increase was greatest in mice that underwent JVB (3,006.86 ± 2,101.61 ng/l), followed by mice that underwent FVB (2,386.50 ± 2,260.98 ng/l) or TVB (2,208.87 ± 1,853.51 ng/l), mice that underwent SVB (1,688.41 ± 811.86 ng/l) or RBB_B (1,643.53 ± 675.49 ng/l) and finally mice that underwent RBB_A (887.62 ± 481.07 ng/l). The increase in corticosterone concentration was significantly smaller in mice that underwent RBB_A than in mice that underwent RBB_B, TVB, SVB, FVB or JVB ($P < 0.05$ for these comparisons).

Open-field test

Latency in the open-field test differed significantly between treatment groups ($F_{5,66} = 4.700$, $P = 0.0010$). Latency was significantly longer in mice that underwent JVB (20.13 ± 24.36 s) than in any other treatment group ($P < 0.05$ for these comparisons). By contrast, latency in mice that underwent RBB_B was the shortest (2.05 ± 2.06 s). Latency did not differ significantly between any other treatment groups ($P > 0.05$ for these comparisons; **Fig. 9**).

Total distance traveled in the open-field test also differed significantly between treatment groups ($F_{5,66} = 8.336$, $P < 0.0001$). Total distance traveled was longest in mice that underwent TVB (2,183.27 ± 348.65 cm) or SVB (2,110.21 ± 443.21 cm) followed by mice that underwent RBB_A (1,807.78 ± 861.96 cm), RBB_B (1,699.41 ± 498.42 cm), FVB (1,225.54 ± 697.78 cm) and finally JVB (839.54 ± 371.71 cm). Mice that underwent JVB or FVB traveled significantly less than

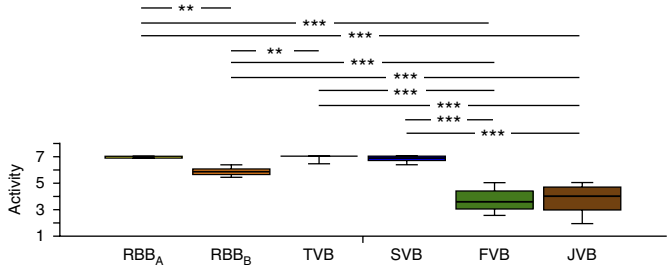

FIGURE 7 | Average in-cage activity level was significantly lower for mice that underwent JVB and FVB than for all other groups. Boxes indicate interquartile ranges, lines inside the boxes indicate medians and whiskers indicate the sample range (minimum–maximum). $^{**}P < 0.05$; $^{***}P < 0.001$.

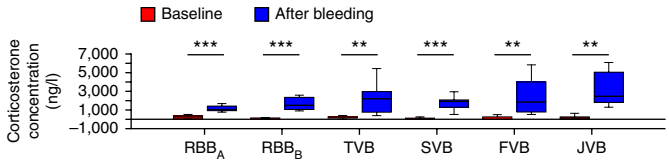

FIGURE 8 | Corticosterone concentration was significantly higher after treatment than before treatment for all treatment groups. Boxes indicate interquartile ranges, lines inside the boxes indicate medians and whiskers indicate the sample range (minimum–maximum). $^{**}P < 0.05$; $^{***}P < 0.001$.

mice that underwent TVB or SVB ($P < 0.05$ for these comparisons), and mice that underwent JVB traveled significantly less than mice that underwent RBB$_A$ ($P < 0.05$). Total distance traveled did not differ significantly between mice that underwent TVB, SVB, RBB$_B$ or RBB$_A$ ($P > 0.05$ for these comparisons; **Fig. 10**).

Average speed in the open field test differed significantly between treatment groups ($F_{5,66} = 8.334$, $P < 0.0001$). Average speed of mice that underwent JVB (2.80 ± 1.24 cm/s) was generally lower than that of any other group and was significantly lower than that of mice that underwent RBB$_A$, TVB or SVB ($P < 0.05$ for these comparisons). Average speed of mice that underwent TVB (7.28 ± 2.16 cm/s) or SVB (7.04 ± 1.48 cm/s) was generally higher than that of any other group. Mice that underwent SVB were significantly faster than mice that underwent FVB or JVB ($P < 0.05$ for both comparisons), and mice that underwent TVB were significantly faster than mice that underwent SVB or JVB ($P < 0.05$ for both comparisons).

Histological examination

At 1 h after RBB$_A$ most indicators of histological change were observed in the retrobulbar connective tissue and Harderian gland. Comparatively fewer indicators of histological change were observed at 1 h after RBB$_B$. At 1 h after TVB most indicators of histological change were observed in subcutaneous connective and adipose tissue. At 1 h after SVB lesions were observed primarily in subcutaneous connective tissue and muscles. At 1 h after FVB indicators of histological change were observed in all examined tissues including the eye. At 1 h after JVB indicators of histological change were observed in muscles and in subcutaneous connective and adipose tissue. At 3 d after RBB$_A$, RBB$_B$, FVB and JVB the incidence of histological change was similar to that observed at 1 h after blood collection. At 3 d after TVB and SVB the incidence of histological change (5.47% and 19.53%, respectively) had decreased relative to the incidence observed at 1 h after treatment (13.97% and 29.41%, respectively; **Table 2**). At 14 d after blood collection no indicator of histological change was observed in most of the samples. In a few samples, indicators of histological change with low qualitative scores were observed (median score = 0 for almost all parameters). The incidence of histological change at this time was very low (<6%) for each treatment group except that which underwent SVB (10.94%).

DISCUSSION

We assessed changes in corticosterone concentration, behavior and histology of mice following six different blood collection methods. Many factors can produce acute stress during blood collection, and we noted potential stressors that differed between methods such as anesthesia (RBB$_B$ and JVB), heating (TVB) and shaving (SVB). These stressors likely interacted with the actual bleeding technique to produce the different physiological, behavioral and histological changes we observed in each group.

The change in corticosterone concentration after blood collection was greatest in mice that underwent JVB, indicating that this group experienced the most physiological stress throughout blood collection. Mice that underwent JVB also had low in-cage activity and long latency, low speed and short total distance traveled in the open-field test. All of these results indicate that JVB was the most stressful method of blood collection with the greatest effect on physiology and behavior in mice.

The change in corticosterone concentration after blood collection was lowest in mice that underwent RBB$_A$, indicating that this group experienced the least physiological stress throughout blood collection. Mice that underwent RBB$_A$ also showed high in-cage activity and short latency, moderate speed and moderate distance traveled in the open-field test relative to other groups. These results indicate that RBB$_A$ was a comparatively less stressful method of blood collection with a small effect on physiology and behavior in mice. This was likely because mice that underwent RBB$_A$ were restrained for the shortest period of time and without anesthesia.

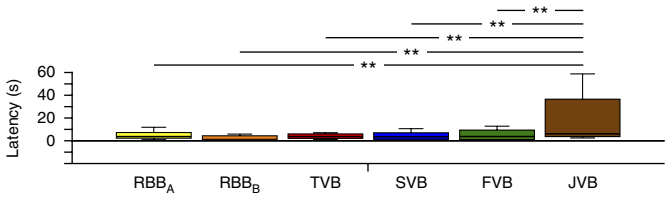

FIGURE 9 | Latency to move toward the edge of the apparatus in the open-field test was significantly longer for mice that underwent JVB than for all other groups. Boxes indicate interquartile ranges, lines inside the boxes indicate medians and whiskers indicate the sample range (minimum–maximum). $^{**}P < 0.05$.

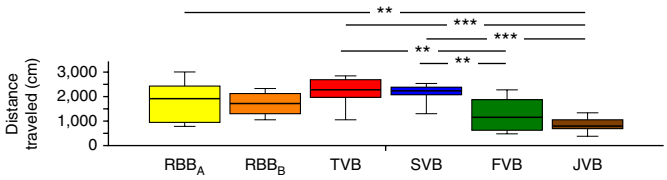

FIGURE 10 | Total distance traveled in the open-field test was significantly shorter for mice that underwent JVB or FVB than for mice that underwent TVB or SVB, and mice that underwent JVB traveled significantly less than mice that underwent RBB$_A$. Boxes indicate interquartile ranges, lines inside the boxes indicate medians and whiskers indicate the sample range (minimum–maximum). $^{**}P < 0.05$; $^{***}P < 0.001$.

Anesthesia probably caused a large amount of stress in mice that underwent JVB or RBB$_B$. By design, our study allows us to compare two very similar techniques, RBB$_A$ and RBB$_B$, which differ primarily in their use of anesthesia. Although RBB$_B$ used a thinner capillary and caused a lower incidence of histological change compared with RBB$_A$, RBB$_B$ involved anesthesia and, correspondingly, mice that underwent RBB$_B$ showed a greater change in corticosterone concentration and lower in-cage activity after blood collection than mice that underwent RBB$_A$. Mice that underwent RBB$_B$ seemed to recover quickly from short anesthesia and performed similarly to mice that underwent RBB$_A$ in open-field tests, but mice that underwent JVB received anesthesia for a longer period of time, which might have contributed to their stronger and longer stress response.

The change in corticosterone concentration was also large and in-cage activity and performance in open-field tests were generally low in mice that underwent FVB compared with all other groups except for mice that underwent JVB. These results indicate that FVB is also a very stressful method of blood collection. FVB took much less time than other techniques, except for RBB$_A$, and did not involve anesthesia, but in-cage activity after blood collection was significantly lower in mice that underwent FVB than in any other group except mice that underwent JVB. This agrees with previous findings that FVB can cause more stress than TVB, SVB or RBB[13,19,44]. Whereas restraint and anesthesia cause stress in other methods, FVB uses no anesthesia and only brief restraint. For this reason, we believe that mice that underwent FVB experienced more stress due to laceration of facial muscles and nerves, even though we used a short lancet.

No previous study has compared all six of the techniques that we used in this study, but previous studies have compared TVB, SVB and FVB, with mixed conclusions. Aasland *et al.*[15] found higher stress in mice after TVB than after SVB, in agreement with our corticosterone measurements after blood collection. By contrast, Madetoja *et al.*[13] found higher stress in mice after SVB than after FVB or TVB, whereas our findings showed lower stress in mice after SVB than after FVB or TVB. This might be explained by the fact that Madetoja *et al.* included a warming period before SVB and a shorter warming period before TVB, whereas we included no warming period before SVB and a longer warming period before TVB. If warming mice before treatment elevates stress levels during blood collection, then the differences between our protocols and those of Madetoja *et al.* could account for the differences in our results.

The change in corticosterone concentration in mice that underwent TVB was similar to that in mice that underwent FVB, even though the time spent on TVB was significantly longer than the time spent on FVB. Mice that underwent TVB also showed significantly higher in-cage activity and faster speed and longer total distance traveled in the open-field test compared with mice that underwent FVB. In our study, TVB and FVB both caused histological changes in subcutaneous connective and adipose tissue, and FVB also caused changes in muscle tissue. A previous comparison found that mice that underwent FVB lost significantly more body weight and had elevated levels of plasma corticosterone after 1 d, compared to mice that underwent TVB[21]. Together with these findings, our results suggest that, despite inducing comparable changes in corticosterone concentrations, FVB causes greater acute stress than TVB and leads to a slower recovery and more extensive histological damage because it punctures a sensitive site.

The incidence of histological change at 14 d after blood collection was very low in all treatment groups except in mice that underwent SVB. This agrees with previous research that compared RBB$_A$ and TVB to JVB[16,25] and compared JVB to TVB[26]. The incidence of histological change at 14 d after blood collection was unexpectedly high for mice that underwent SVB for reasons that are not known. We generally found few changes during histological examination, and we consider these associated effects of blood collection to be of less concern and importance than our measured indicators of stress.

TABLE 2 | Incidence of histological change after blood collection

Bleeding technique	1 h after	3 d after	14 d after
RBB$_A$	15.08%	15.87%	4.37%
RBB$_B$	5.95%	7.54%	3.17%
TVB	13.97%	5.47%	5.47%
SVB	29.41%	19.53%	10.94%
FVB	12.50%	13.13%	5.63%
JVB	9.56%	10.16%	3.91%

During procedures that involve blood collection, researchers should try to minimize restraint time and routine handling to avoid unnecessary stress[15]. They should also minimize acute stress from pain and possible tissue damage. Depending on the focus and requirements of an experiment, researchers must carefully select a blood sampling method from among the established options, giving consideration to the different effects that we observed in this study.

Our results suggest that FVB and JVB led to higher acute stress and that RBB_A and RBB_B caused less stress than other techniques, based on corticosterone concentrations, behavioral indicators of stress and incidences of histological change after blood collection. Although mice that underwent TVB and SVB had higher incidences of histological change at 14 d, they also had relatively lower corticosterone concentrations and low behavioral indicators of stress, indicating that these methods are also acceptable. We note that only small volumes of blood were collected in this study. These findings might not apply to procedures for collecting greater volumes of blood, as these could require longer restraint and cause higher stress and incidence of histological changes in mice.

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The authors declare no competing financial interests.

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