NOTE

Laboratory Routines Cause Animal Stress

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Eighty published studies were appraised to document the potential stress associated with three routine laboratory procedures commonly performed on animals: handling, blood collection, and orogastric gavage. We defined handling as any non-invasive manipulation occurring as part of routine husbandry, including lifting an animal and cleaning or moving an animal's cage. Significant changes in physiologic parameters correlated with stress (e.g., serum or plasma concentrations of corticosterone, glucose, growth hormone or prolactin, heart rate, blood pressure, and behavior) were associated with all three procedures in multiple species in the studies we examined. The results of these studies demonstrated that animals responded with rapid, pronounced, and statistically significant elevations in stress-related responses for each of the procedures, although handling elicited variable alterations in immune system responses. Changes from baseline or control measures typically ranged from 20% to 100% or more and lasted at least 30 min or longer. We interpret these findings to indicate that laboratory routines are associated with stress, and that animals do not readily habituate to them. The data suggest that significant fear, stress, and possibly distress are predictable consequences of routine laboratory procedures, and that these phenomena have substantial scientific and humane implications for the use of animals in laboratory research.

Stress induced by highly invasive procedures used in some animal studies is well recognized by regulatory authorities and Institutional Animal Care and Use Committees. Animals used in laboratory research and testing are also regularly subjected to routine maintenance or monitoring procedures, such as personnel entering the animal housing room, cage movement and cleaning, body weight collection, physical examination, injections, and collection of blood or other tissues. Because these procedures may be considered incidental in nature, their effects on laboratory animal well-being may be overlooked by ethical review committees, whose task is to try to reduce the potential for animal pain and suffering. Some studies suggest that routine procedures may cause significant stress to animals, and that even seemingly benign animal studies may raise substantial animal welfare concerns.

This paper summarizes findings from other studies, focusing on three procedures commonly performed on animals used in laboratory research: routine handling, blood collection, and orogastric gavage. We address the possibility that routine procedures are intrinsically stressful to laboratory animals regardless of the care and skill with which they are performed. Throughout this paper, we define *stress* as "the effect produced by external (i.e., physical or environmental) events or internal (i.e., physiologic or psychologic) factors, referred to as stressors, which induce an alteration in an animal's biologic equilibrium" (1). We define *distress* as "an aversive state in which an animal is unable to adapt completely to stressors and the resulting stress and shows maladaptive behaviors" (1).

Methods

Two online databases (PubMed and NLM Gateway) were used to identify studies published in English involving quantitative or behavioral measures of pain or stress in animals during routine laboratory handling, blood collection, and orogastric gavage. The following root terms were used during searches: animal, laboratory, stress, rat, mouse, primate, bird, cat, dog, handling, venipuncture, venepuncture, bleeding, gavage, force-feeding, and oral dosing. PubMed contains articles dating back to 1966, and NLM Gateway includes articles from 1953 to 1965. Other papers were found by scanning literature cited in retrieved papers. "Handling" was defined as any non-invasive manipulation that is part of routine husbandry, including lifting an animal, cage cleaning or moving an animal's cage.

For reported physiologic measurements, we calculated the difference between the mean baseline or control measure and the mean maximum measure following the intervention and converted this to a percentage change from baseline. For variables decreasing in response to stressors, mean minimum measures were used. This difference was then divided by the baseline or control measure, and the quotient multiplied by 100 to derive percentage.

Results

Handling. (i) Rats and mice. A summary of studies reporting animal responses to routine handling procedures is presented in Table 1. Heart rate (HR) and blood pressure (BP) were shown to increase in both male and female rats monitored by telemetry in response to a variety of handling procedures, including animal lifting, cage changing, simulated cage changing (rat returned to original cage), restraint with subcutaneous injection, transport to another room with subcutaneous injection, restraint with tail vein injection, being present in the room during decapitation of other rats, handling and body weight collection, and handling and vaginal lavage (2-5). These responses persisted for 45 to 90 min following the procedures. Mean HR rose significantly in adult male rats removed from their home cages once daily for 20 days and placed in an adaptation chamber for either 2-min (n = 13) or 1-h (n = 7) sessions. Although HR gradually returned to near resting levels by the end of the 1-h sessions, animals showed no signs of habituating to the handling over the course of the experiment as judged by latency of HR to return to baseline (6). A rise in core body temperature of 1°C occurred within 30 min and lasted several hours in each of five male rats after being handled three times for 30 sec for insertion of a rectal temperature probe (7).

During cage changing, physiologic perturbations were similar whether rats were picked up and placed in new cages or were returned to their original cages. This suggests that animal responses are caused by physical manipulation rather than by unfamiliar aspects of new surroundings, such as new bedding (4). Placement of soiled bedding in new cages did not reduce stress responses (5).

Routine handling has been shown to elevate serum concentrations of hormones such as corticosterone, which mediates responses to stress in addition to regulating protein and carbohydrate metabolism, and prolactin, which also mediates stress responses. Blood samples col-

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Table 1. Summary of published findings on animals' response to handling

Study	Animals	N	Procedure	Parameter measured	Calculated maximum % change[a]	*P*
Sharp et al. 2002b	male rats	8	cage change	heart rate	+46%	≤ 0.05
				blood pressure	+19%	≤ 0.05
Sharp et al. 2001	female rats	8	cage change	heart rate	+37%	≤ 0.05
				blood pressure	+15%	≤ 0.05
Sharp et al. 2003a	female rats	7-8	cage change	heart rate	+37%	< 0.05
				active behaviors	+190%	< 0.05
			cage change simulated	heart rate	+30%	< 0.05
				active behaviors	+223%	< 0.05
			handled and weighed	heart rate	+46%	< 0.05
				active behaviors	+441%	< 0.05
Duke et al. 2001	male rats	6-8	cage change	heart rate	+20%	< 0.05
				blood pressure	+34%	< 0.05
Gallaher et al. 1985	male rats	5	handled[b]	body temperature	+2.7%	NA
Armario et al. 1986a	male rats	6	handled	corticosterone	+300%	< 0.0001
			cage moved	glucose	+9%	< 0.05
Barrett and Stockham 1963	male rats	12	handled	corticosterone	+338%	< 0.001
Gärtner et al. 1980	male rats	8	cage moved	corticosterone	+63%	< 0.001
				prolactin	+83%	< 0.001
				TSH	+90%	< 0.001
				T3	+42%	< 0.001
				packed cell volume	+6%	< 0.05
				haemoglobin	+11%	< 0.05
				protein	+25%	< 0.01
De Boer et al. 1990	male rats	6	handled	corticosterone	+160%	< 0.05
				noradrenaline	+290%	< 0.05
				adrenaline	+220%	ns
				glucose	+195%	ns
Brown and Martin 1974	male rats	15	novel environment	corticosterone (crest)[c]	+31%	NA
				corticosterone (trough)[c]	+200%	NA
				prolactin (crest)	+140%	NA
				prolactin (trough)	+135%	NA
				growth hormone (crest)	+550%	ns
				growth hormone (trough)	+465%	ns
Zethof et al. 1993	male mice	320	handled	body temperature	+3.1%	< 0.001
Seggie and Brown 1974	male mice	355	handled	corticosterone (crest)	+190%	< 0.01
				corticosterone (trough)	+175%	< 0.01
				prolactin (crest)	+275%	< 0.001
				prolactin (trough)	+220%	< 0.001
				growth hormone	+400%	< 0.001
Tabata et al. 1998	male mice	6	cage moved	glucose	+30%	< 0.01
	female mice	6	cage moved	glucose	+24%	< 0.05
	male rats	6	cage moved	glucose	+1%	ns
	female rats	6	cage moved	glucose	+3%	ns
Clement et al. 1989	male mice	4	handled	body temperature	+4.8%	NA
Moynihan et al. 1990	male mice	36	handled	immunoglobulin (IgM)	–26%	< 0.05
				immunoglobulin (IgG)	–28%	< 0.05
				lymphocyte CD4 (L3T4)	–7.0%	ns
				lymphocyte CD8 (Lyt2)	+31%	ns
				lymphocyte Pan T (Thy 1)	+8.5%	ns
				lymphocyte IgM	+6.6%	ns
Moynihan et al. 1989	female mice	20	handled	immunoglobulin (IgG)	+37%	< 0.05
				immunoglobulin (IgM)	NA	ns
Solomon and Cohen 1968	mice	108	handled	primary antibodies	+100%	< 0.001
				secondary antibodies	+118%	< 0.02
Newton et al. 1962	rats	48	handled	post-challenge survival	+15%	< 0.01
Line et al. 1989	female rhesus macaques	6	cage change	heart rate	+46%	NA

Table 1. Summary of published findings on animals' response to handling (cont.)

Study	Animals	N	Procedure	Parameter measured	Calculated maximum % change[a]	*P*
Capitanio et al. 1996	male rhesus macaques	37	room entered	leukocyte (CD8) count	+50%	< 0.05
Meyer and Knobil 1967	rhesus macaques	3	various[d]	growth hormone	0 – +2,400%	NA
Matt et al. 1983	female hamsters	12	cage moved	prolactin	+125%	< 0.05
Conn et al. 1989	hamsters	12	cage change	body temperature	+3.7 – 4.6%	< 0.005
Knudtzon 1984	rabbits	5	cage moved	glucose	+21%	< 0.01
				glucagon	0%	ns
				insulin	–19%	ns
Widmaier et al. 1994	fruit bats	195	handled	cortisol	+100%	< 0.001
				corticosterone	+150%	< 0.02
Le Maho et al. 1992	male geese	7	handled	corticosterone	+160%	< 0.01
				epinephrine	+165%	< 0.01
				norepinephrine	+160%	< 0.01
Romero and Remage-Healey 2000	starlings	18	handled	corticosterone	+100 – 350%	< 0.0001
Dawson and Howe 1983	starlings	57	handled	corticosterone	+400%	< 0.001
Harvey et al. 1980	ducks	90	handled	corticosterone	+385%	< 0.001
Beuving and Vonder 1978	hens	14	handled	corticosterone	+550%	< 0.05
Romero et al. 1997	sparrows	82	handled	corticosterone	+260 – 730%	< 0.0001

[a]from baseline or control.
[b]with insertion of rectal probe.
[c]of diurnal adrenal gland cycle.
[d]agitation (not defined), room entry by unfamiliar persons, telephone ringing, abdominal skin pinched forcefully.
ns, statistically non-significant.
NA, statistical significance not provided by cited authors.

lected from adult rats 15 min after a 15-sec period of handling showed significant elevations in concentrations of serum corticosterone (8, 9) and prolactin (9), which persisted for 30 to 60 min. Stress-like hormonal responses varied with the intensity of handling stressors. Cage change with transfer to a quiet room was more stressful than cage change without transfer, while cage change and transfer to a noisy room elicited the greatest hormonal elevations (9). Corticosterone and prolactin elevations following handling were pronounced at both the crest and trough of the diurnal adrenal gland cycle in rats (10, 11). Being handled for 5 sec, or being placed for 3 min in a novel environment (open-topped cardboard drum 65 cm high and wide) caused corticosterone and prolactin levels to "respond dramatically" in individually housed male rats bled by decapitation (10).

Plasma corticosterone quadrupled within 15 min, and decreased significantly 30 to 60 min later in 12 male rats in response to brief routine handling and weighing. Rats bled after being transported to a laboratory also had threefold higher plasma corticosterone levels than did rats bled in their holding room (12). Significant increases in mean plasma corticosterone and noradrenaline concentrations were reported in six individually housed, catheterized male rats in response to being lifted gently from their cage, held over another cage then returned to their home cage, or being lifted and placed for 15 min in a similar clean cage lacking bedding, food, or water (13).

Serum corticosterone levels rose significantly in eight adult male rats within 5 min of moving cages from a shelf to the floor without opening lids, peaking at 15 min and remaining above baseline up to 60 min later (14). Levels of prolactin, TSH (thyroid-stimulating hormone), and T3 (triiodothyronine) also rose significantly within 15 min and were still increasing up to 1 h later. Other blood indices, including packed cell volume, hemoglobin, and plasma protein, increased significantly within 2 min of handling, peaking at about 5 min, and reverting to baseline by about 10 min (14).

Stress stimulates the body to release glucose into the bloodstream in physiologic preparation for the "fight or flight" response. Plasma glucose concentrations increased in proportion to handling time in 40 adult male rats bled by cardiac puncture or decapitation (15), but did not rise significantly in 12 rats following handling and transport to an adjoining room (16). Growth hormone decreased markedly in male rats (n = 355) in response to being lifted and held for 5 sec (11).

Research suggests that mice are at least as sensitive as rats in their responses to potential laboratory handling stressors. Personnel entering the animal room, animal handling, and cage cleaning all caused acute increases in heart rate and body temperature in mice studied by telemetry (18). Mice habituated to personnel entering the holding room after 2 weeks, but not to handling (18). Plasma glucose concentrations also rose significantly in 12 mice following handling and transport to an adjoining room (16).

Body temperatures increased approximately 1.7°C in four male mice monitored by telemetry after being picked up and restrained for 15 sec as if for injection. In these same animals, body temperatures peaked 15 min post-handling and remained elevated for several hours (19). Animal activity also increased significantly during the first hour following handling, potentially contributing to the elevation in body temperature. Stress-induced hyperthermia, a phenomenon in which the last remaining animal from a cohort of group-housed animals removed sequentially from a home cage demonstrates the greatest elevation in body temperature, appears to be a robust and reproducible phenomenon in mice (20, 21). Stress-induced hyperthermia was observed in 12 separate groups of 10 mice, in which animals were successively handled for rectal temperature collection. Temperatures returned to baseline in 30 to 60 min (21).

Mice restrained manually for 10 or 20 sec showed no significant

change from normal behavior levels (measured as a "disturbance index" based on exploratory activity levels) when released into a familiar observation cage, but mice held for 30 or more sec showed a statistically significant change in activity. This suggests that handling duration may be an important variable in inducing stress in mice (22).

Like rats, mice also show pronounced corticosterone level changes in response to being handled. Corticosterone levels rose significantly in both group-housed (five per cage, n = 10) and individually housed adult male mice removed from their cages, placed for 1.1 h in a black Plexiglas chamber, and then bled following decapitation. The mice failed to habituate to handling over a 15-day period (23).

The relationship between handling and immune response is complex. Different stressors can affect the same parameters of the immune system quite differently, and for a given stressor some parameters may show alterations while others do not (24). Individually housed male C3J/HeJ mice (n =18) handled daily for 2 weeks by being restrained as if for an injection developed significantly lower antibody titers of both immunoglobulin M (IgM) and immunoglobulin G (IgG) to the antigen KLH (keyhole limpet hemocyanin) than did 18 unhandled controls (25). Primary IgG (but not IgM) response to intraperitoneally (i.p.)-injected KLH was reduced at 5, 10 and 15 days post-challenge in three replicates of experiments in which 12 group-housed female mice were gently handled once daily without restraint for 2 weeks (26). Levels of lymphocyte cell surface markers CD4 (L3T4), CD8 (Lyt2), pan T (Thy1) and IgM did not differ in splenocytes collected from mice handled for 1 or 2 weeks, or unhandled controls (26).

In each of three replications of another experiment, DBA/2 mice handled daily (removed from home cage, placed in a small container for 3 min then returned to the home cage) from birth to 24 days had significantly lower survival rates following i.p. implantation of leukemia cells compared with unhandled controls (27). A similar protocol with BALB/c mice found no differences in survival rates between handled and unhandled groups (28).

Fisher rat pups (n = 54) placed in boxes for 3 min a day from birth until weaning (21 days) developed higher primary and secondary serum antibody titers to the novel antigen flagellin than did 54 unmanipulated controls (29). Holzman rats (n = 24) held and stroked for 10 min daily from weaning (22 days) to 45 days survived significantly longer (mean 33 days) following subcutaneous injection of cancer cells (Walker carcinoma 256) than did unhandled controls (n = 24; mean 28.7 days) (30).

The relationship between handling and immune response is not necessarily linear in mice. Tumors grew faster in female mice (n = 24) handled daily (picked up by the tail and held gently in the palm for 2 min for 2 weeks prior to tumor implantation), and slower in animals handled for only 1 week (31). Fifteen female mice subjected to daily handling (lifted and cupped gently in the hand for 2 min) on days 1 through 5 had increased tumor size compared to non-handled controls; however, 13 mice handled on days 1 through 7 following tumor implantation showed no change in tumor growth rate (32).

There is evidence that handling effects on murine immunity are not mediated by alterations in glucocorticoid responses. No difference in baseline corticosterone levels was found between male C3H/HeJ mice handled daily and unhandled controls despite a lower IgM and IgG antibody response in the handled animals (25). Similar results in antibody response were noted in handled male BALB/c mice (n = 12). In addition, there were no changes in glucocorticoid levels in handled BALB/c mice following i.p. injection of KLH, whereas previously unhandled mice (n = 12) had significantly elevated corticosterone levels in response to the same injection (31).

(ii) Nonhuman primates. Limited data in nonhuman primates suggests that routine handling causes alterations in a number of physiologic parameters indicative of stress, including heart rate, growth hormone levels, and leukocyte responses. Heart rates of six rhesus macaques increased markedly (to 175 bpm from resting rates of 96-149 bpm) in response to cage change, and remained elevated for 2 h after the procedure (33). Growth hormone may be influenced by many factors, including stress (34), and was found to increase abruptly in two of three chronically catheterized female rhesus macaques in response to agitation (not defined) and room entry by unfamiliar persons, and in all three monkeys in response to the onset of a telephone ringing (35). Levels of CD8+ T lymphocytes, a critical sub-population of regulatory T-lymphocytes, were approximately 50% higher in singly housed adult male rhesus macaques (n = 37) bled in squeeze-back cages ≥ 6 min after initial room disturbance, than in macaques bled within 5 min (36).

(iii) Other mammals. Stress related to routine handling procedures has been reported in limited studies of other mammals used in laboratory research. Routine experimental manipulations, including animal transportation, induced significant but variable elevations in serum prolactin levels in 18 female hamsters (37). Mean body temperature of both sedentary and exercised hamsters (n = 12) rose significantly and peaked within 1 h of cage changing (38). Seven female New Zealand White rabbits had significantly elevated blood glucose (but not glucagon and insulin), which required up to 2 h to return to baseline, following cage transportation (39).

Significant increases in plasma cortisol and corticosterone levels occurred in two species of wild-caught Old World fruit bats (*Pteropus hypomelanus* and *P. pumulis*, n = 19) twice daily following 10-15 min of routine handling, transport, and observation (40). Routine non-invasive daily handling procedures (e.g., body weight recording, measuring) also resulted in significant increases in plasma cortisol and glucose levels in three Old World fruit bats *P. hypomelanus*, *P. vampyrus* and *Rousettus aegyptiacus* (41).

(iv) Birds. Capture and handling are known to be stressful for free-living birds, and are accompanied by rapid release of corticosterone from the adrenal glands (42). At all times of the day and across all seasons, blood corticosterone levels increased markedly in 18 captive wild starlings handled and restrained for 15, 30, or 45 min (43). Following capture with mist nets, plasma corticosterone concentrations increased to fourfold baseline within 2 min post-capture in 57 starlings bled following decapitation (44).

Corticosterone levels increased significantly within 45 sec in hand-restrained hens (45) and within a few minutes of capture in white-crowned sparrows (46). Considerable individual variation in corticosterone response to handling was observed in hens sampled through an indwelling catheter. Some hens barely reacted, while others reacted profoundly (up to 1,000% increase from baseline after 7 min) (45). The authors noted that some of the more extreme responders might have experienced hypovolemic shock due to oversampling. In the same study, another experiment found that elevations in hen corticosterone levels did not diminish with repeated handing (4 times daily for 5 days) (45). Corticosterone levels increased rapidly in 8-week old domestic ducks bled 14 times from the brachial vein at 1-min intervals (47), though lack of detail in the description of bleeding method precludes determining the relative contributions of handling and venipuncture to these birds' stress response.

Corticosterone levels increased more than 150% in male geese (n = 7) placed for 15 min in boxes that limited wing movement but permitted free movement of the head and neck (48). These birds, monitored remotely by surgically implanted catheters during a 2-week period of experiments, at first appeared frightened, flapping their wings or trying to escape. Later, they looked very quiet, with only slight trembling detectable upon close observation. These behavioral changes were not accompanied by significant declines in corticosterone

Table 2. Summary of published findings on animals' response to blood collection

Study	Animals	N	Procedure	Parameter measured	Calculated maximum % change[a]	*P*
Haemisch et al. 1998	male rats	10	bled from tail tip	corticosterone (crest) corticosterone (trough)	+62% +366%	< 0.05
Vachon and Moreau 2001	male rats	10	cannulated or anesthetized	corticosterone	+118%	< 0.01
Bickhardt et al. 1983	male rats	48	decapitation	protein	NA	< 0.01
Tuli et al. 1995	male mice	10	bled from tail tip	corticosterone	+595%	< 0.05
Tabata et al. 1998	male mice female mice	10 10	bled from tail tip bled from tail tip	glucose glucose	+44% +64%	< 0.01 < 0.01
Knudtzon 1984	female rabbits	7	bled from ear vein	glucose	+60%	< 0.05
Heding and Andersen 1973	rabbits	6	bled from ear vein	glucose	+24%	NA
Drouhault et al. 1983	rabbits	6	bled from ear vein	glucose	+120%	< 0.05
Elvidge et al. 1976	female rhesus macaques	18	bled from leg vein	cortisol	+40%[b]	< 0.001
Reinhardt et al. 1990	female rhesus macaques	25	bled from leg vein	cortisol	+50%	< 0.001
Reinhardt et al. 1991	rhesus macaques	6	bled from leg vein	cortisol	+52%	< 0.025
Reinhardt 1996	rhesus macaques	10	bled from leg vein	cortisol	+63%	NA
Herndon et al. 1984	rhesus macaques	1	bled from leg vein	cortisol	NA	< 0.01
Dettmer et al. 1996	capuchin monkeys	8	bled from leg vein	cortisol	+66%	< 0.05

[a]from baseline or control.
[b]trained, unanesthetized group only.
NA, statistical significance not provided by cited authors.

levels, and the authors concluded that a quiescent appearance in geese is not sufficient evidence that the animal is not stressed (48).

Blood collection. Blood collection is one of the most common procedures conducted on laboratory animals. Methods vary both within and among species used. In rodents, the tail vein, jugular vein, or retro-orbital sinus are commonly used venipuncture sites. In non-human primates, the saphenous vein is often used, and in rabbits, blood is commonly removed from the marginal ear vein. Other methods, used primarily in rodents, include tail tip excision, cardiac puncture, and decapitation. During blood collection procedures, stress may result from pain, routine handling and restraint. An exception may occur with the use of chronic indwelling catheters, which require an initial minor surgical procedure. Chronically catheterized animals are often tethered, which may induce stress; because animals often try to remove catheters, they may be subjected to additional restraint (e.g., jackets, Elizabethan collars). Table 2 presents a summary of studies reporting animal responses to blood collection.

(i) **Rodents and lagomorphs.** Common methods of blood sampling were associated with increases in corticosterone levels in rats and mice. Corticosterone levels rose significantly in five conscious cannulated male rats and in five isofluorane-anesthetized non-instrumented male rats within 15 min following bleeding via the jugular vein (49). Corticosterone levels in the conscious animals rose further by 30 min and returned to baseline by 60 min, while in the anesthetized animals these levels took more than an hour to subside, likely due to the anesthetic agent.

Repeated blood sampling (at 20, 60, and 120 min) from the tail caused significant increases in corticosterone levels in 10 male rats during both diurnal (0800 to 1000 h) and nocturnal (1800 to 2000 h) sampling sessions (50). Levels were greater than baseline at 20 min and 60 min, and had returned to baseline 120 min post-procedure. Mean beta-endorphin levels also peaked at 20 min, and remained significantly elevated for the remainder of the sampling session (50).

Similar to handling, blood collection has also been shown to induce elevations in blood glucose. Glucose levels rose significantly in seven New Zealand White rabbits bled (4 ml) at 2-h intervals for 15 h or 32 h (39), in six Fauve de Bourgogne rabbits bled (3 ml) hourly for 4 h (51), and in six albino Rex rabbits bled (amount unreported) three times in one hour (52). All of the rabbits in these studies were fasted prior to bleeding and all were bled from the marginal ear vein.

Certain blood sampling methods warrant particular mention from a humane standpoint. Probably most controversial is collection of blood from the retro-orbital sinus of the eye in rodent species. This technique involves restraining the animal firmly by the scruff of the neck, usually under sedation or anesthesia, then penetrating the orbital sinus by advancing a sterile micro-pipette or micro-capillary tube through the medial conjunctiva to the back wall of the orbit to puncture the venous sinus (53). Following withdrawal of the pipette, pressure may be applied with a soft compress to limit hemorrhage after the sample has been obtained.

Numerous problems and complications are associated with retro-orbital venipuncture, including excessive pressure on the eye; damage to the optic nerve and other intra-orbital structures leading to visual deficits and blindness; increased pressure leading to proptosis, ulceration, and rupture; and infection leading to ocular inflammation and subsequent degeneration (53). Abrasion of the cornea following pressure-induced hemostasis may result in keratitis and neovascularization. These in turn may lead ultimately to a non-functioning "sunken eye" (enophthalmia) with the animal experiencing "considerable pain in the interim period" (53). Additional complications include hemorrhage, abscess, and damage to the globe (54).

In a study in which 204 rats were bled from the retro-orbital sinus, all four participating animal technicians, each with greater than 10 years experience, induced ocular discharge; three of the technicians induced at least one case of both enophthalmia and exophthalmia (the fourth caused enophthalmia only), two induced corneal lesions,

and three induced intraocular alterations in at least one animal (55). During a study of cholesterol metabolism, a nonsignificant trend towards increased enophthalmia was reported in the eyes of 36 male rats sampled by retro-orbital bleeding compared with no cases in their other unsampled eyes (56).

Significantly higher levels of prolactin were determined in 15 male rats sampled daily for one week by retro-orbital bleeding than in 15 male rats bled via the sublingual vein (57). Retro-orbital blood samples also had significantly higher levels of creatine kinase and aspartate aminotransferase, suggesting that more tissue is damaged with this method, a finding also reported in other studies (58, 59). Repeated anesthesia and blood sampling on the same day also induced body weight loss and depressed food consumption (57). A massive stress response to diethyl ether was thought to mask any stress induced by retro-orbital sampling in a study using 12 male rats (60). While corticosterone, adrenaline and glucose levels were similar in the non-sampled and sampled animals, plasma levels of noradrenaline were significantly higher in the sampled group 15 min after anesthesia, suggesting a response to tissue damage (60).

Collecting blood from rats or mice by tail tip incision has become an established procedure in many institutes working with transgenic rodents (54). Repeated excision of the tail tip results in trauma to cartilage and eventually to the coccygeal vertebrae, and is considered too painful to be carried out in a conscious rat (61). "Milking" the tail may also induce an inflammatory leucocytosis, believed by some to be painful (61). Blood sampling of 10 mice by partial sectioning of the tail vein induced a rapid increase in plasma glucose levels that required an hour to return to baseline (16). Corticosterone levels rose significantly in five mice immediately following tail vein blood sample collection compared to levels in five mice bled immediately following cervical dislocation (62).

Collecting blood by cardiac puncture can also be associated with problems, including pericardial hemorrhage, leading to cardiac arrest and bleeding into the thorax (53). Death rates up to 12% have been associated with this procedure in some studies (63). The procedure should only be conducted under anesthesia and it should only be used for terminal blood sampling (61).

(ii) Nonhuman primates. Common behavioral responses to blood sampling in nonhuman primates include fear-grinning, vocalizations, diarrhea, and physical resistance (e.g., struggling, refusing to enter a squeeze cage) (64). Evidence from a number of studies suggests that rewards can mitigate stress responses.

Cortisol levels rose significantly after 15 min in 10 rhesus macaques removed from their home cages for restraint and venipuncture, compared with 15 animals sampled using a squeezeback mechanism in their homecage and immediately given a food reward (65). Blood cortisol levels increased significantly (52%) 15 min after six singly caged adult male rhesus macaques were bled in a restraint apparatus outside their cage, but not (13%) when they were bled in their home cages (66). While cortisol levels were significantly higher in six untrained rhesus macaques bled three times from the leg vein over a 2-h period than in six trained animals handled in the same fashion, cortisol levels also increased significantly by up to 40% over the same period in the trained group (67).

Serum growth hormone concentrations were significantly increased in four male rhesus macaques restrained and then bled via the saphenous vein (68). In a fifth monkey, increases in serum cortisol levels were significant following venipuncture with restraint outside the home cage, but not when blood was drawn remotely in the home cage via an indwelling venous catheter.

Eight capuchin monkeys demonstrated partial adaptation to restraint and venipuncture (69). All animals showed increased average cortisol levels compared with baseline following thrice weekly blood samplings over 6 weeks. After 19 days of training, no monkey presented a leg or an arm for venipuncture, and only four were scored as being behaviorally habituated to the procedure.

(iii) Dogs. Data on the effects of blood collection in dogs are limited, but they are similar to those for nonhuman primates. Plasma renin activity, epinephrine concentrations, and hematocrit values changed significantly and required at least 30 days to stabilize in four female beagles bled on days 1-22 and 30-41 using either indwelling catheters or daily venipuncture techniques (days 2, 15, 34, and 41) (70). One of the four dogs exhibited behavioral signs of fear—freezing and low posture—early on during the study. This behavior diminished somewhat by day 30 but remained evident throughout the course of the study.

No significant procedural effect was found in a study of six male beagle dogs bled four times daily for 4 days using either repeated venipuncture or indwelling catheters (controls) (71). The concentrations of the three measured hormones (cortisol, luteinizing hormone, and testosterone) varied considerably within and among dogs. The authors reasoned that the most likely explanation for the dogs not being measurably stressed was that they were familiar with the procedures and their professional handlers (71).

Orogastric gavage. Orogastric gavage is a common laboratory method in toxicology and pharmacokinetic studies, where it is commonly used for daily dosing of rats, mice, rabbits, and monkeys (72-74).

Gavage involves the physical stresses of handling and restraint, insertion of a rigid metal or flexible plastic tube (typically 10 cm long × 2 mm diameter) from mouth to stomach (with associated breathing interference), and stomach distension. Other potential stressors include possible toxic effects of the agent being studied and physiological effects of the liquid vehicle used to facilitate delivery and absorption (61, 72). Complications include inadvertent tracheal administration, reflux, aspiration pneumonia, esophageal impaction, trauma or perforation, hemothorax, and death (72, 75). More viscous vehicles, such as oils, appear to be more stress-inducing than less viscous vehicles (e.g., water), which in turn have the apparent disadvantage of increased risk of aspiration pneumonia. Table 3 summarizes findings from five studies involving orogastric gavage in rats.

Several recent studies have assessed the potential pain, stress, and distress associated with orogastric gavage in animals. Many of the complications listed above are associated with restraint and incorrect placement of the tube, though we did not identify any studies specifically assessing this. One survey suggested that the "ideal" orogastric gavage dose volumes in rats should be ≤ 10 ml/kg (75). Although these are not recommended in regulatory guidelines, volumes as high as 20-50 ml/kg are sometimes administered (76). Upper limits for oral dosing in the mouse and the rat are 50 and 30 ml/kg, respectively. Above these volumes, stomach distension is noticeable (77). Increasing dose dilution was associated with increased toxicity (reflected in higher mortality rates) in rats gavaged with 12 different organic acids and bases, and inorganic compounds. The authors of this study attributed this effect to higher absorption rates (78).

Gavage-induced stress appeared to cause massive hepatic apoptosis in 88 rats dosed daily with 120 mg/kg (10 ml/kg) of cyproterone acetate for 10 days (79). A sham-treatment group (sham gavaged with a dry needle) showed similar physiological outcomes, suggesting that gavage itself was inducing the hepatic pathology.

Anatomic damage was also documented in a 2-year carcinogenicity study (compound not mentioned) in which 200 male and 200 female Fisher 344 and Sprague-Dawley rats were gavaged daily using a 0.4 cm diameter rigid metal gavage tube (80). By week 45, 64 of the Fisher female rats (32%) and 13 of the Fisher male rats (6.5%) had died. Necropsies suggested death occurred by asphyxiation following obstruction of the oropharyngeal cavity by a bolus of impacted

Table 3. Summary of published findings on animals' response to gavage

Study	Animals	N	Procedure	Parameter measured	Calculated maximum % change[a]	*P*
Roberts et al. 1995	rats	88	sham (dry tube insertion)	liver apoptosis (% of hepatocytes)	+355%	≤ 0.01
Germann and Ockert 1994	female rats	200	aqueous solution	death[b]	+24%	NA
Murphy et al. 2001	female rats	9	awake	weight loss	+9%	< 0.05
		9	awake	death[b]	+56%	NA
		37	halothane anesthetized	weight loss	+3.5%	< 0.05
		37	halothane anesthetized	death[b]	+3%	NA
Brown et al. 2000	male rats	10	corn oil[c]	corticosterone	+596%	< 0.05
			water[c]	corticosterone	+58%	ns
			MCT80[d]	corticosterone	+350%	ns
Alban et al. 2001	male rats	41	$BaSO_4$	ambulation	–39%	< 0.001
				rearing	–53%	< 0.04
				defecation	undefined[e]	< 0.013
				body temperature	–2.8%	< 0.014

[a]from baseline or control.
[b]percentage mortality of treatment group animals.
[c]maximum (40 ml/kg) dose compared with sham (dry insertion tube).
[d]maximum (30 ml/kg) dose compared with sham (dry insertion tube).
[e]mean defecation was zero at maximal doses, therefore percentage change is mathematically undefined.
ns, statistically non-significant.
NA, statistical significance not provided by cited authors.

food or bedding. The authors believed these obstructions were exacerbated by the development of granulation tissue in the oropharynx following repeated gavage (80).

An "unacceptably" high incidence of gavage-related respiratory distress (22%), significant (> 5%) weight loss beyond day 5, and death (56%) were observed in nine rats gavaged daily (5 ml/kg) for 10 days without anesthesia (72). Esophageal trauma, induced by conscious animal resistance to passage of the gavage needle, was thought to significantly contribute to mortality in this study. Two animals were euthanized due to respiratory distress and severe (13%) weight loss, and four rats died from gavage-related effects (not specified). In comparison, one of 37 (3%) animals died when given brief inhalation anesthesia prior to orogastric gavage (72).

In rats administered water, Tween 80 (a non-toxic surfactant), and various food-grade oils, both the vehicle and the volume influenced stress responses (75). Despite pre-study acclimation to daily restraint and insertion of a dry gavage needle, rats administered corn, sesame, soybean, or peanut oil as vehicles demonstrated a dose-dependent increase in serum corticosterone levels over a 24-h period. These levels were significantly higher than those of sham-gavaged rats in all but the soybean oil group. Other signs of pain and distress included vehicle reflux, diarrhea, foamy fluid in the trachea, edematous lungs, and gastric distension. Large doses of the less viscous vehicles (water and Tween 80) resulted in aspiration with subsequent airway and lung damage. Aspiration was observed in 20% to 80% of conscious animals gavaged at ≥ 30 ml/kg (75).

In a study using 41 male rats, fasted for 24 h prior to gavage (water provided ad libitum), animals dosed with volumes ≥ 12 ml "showed some discomfort (undescribed) during dosing," and animals were noted to move, rear, and defecate significantly less with increasing dose volumes (74). Reductions in post-gavage body temperatures also correlated with increasing dose volume. Cyanosis was also commonly observed following gavage, likely due to hypoxia from the extended restraint (in plastic tubes) during the procedure.

Witnessing effects. Several studies have addressed the possibility that merely witnessing other animals being subjected to procedures like those described above might be stressful. For example, being in a room where other rats were subjected to routine cage changes induced significant increases in HR and BP in male rats housed alone or with one cagemate (81). Similarly, HR increased significantly in singly housed female rats that were present when other rats were being handled and weighed, and in both single, paired, and group-housed females that were present when other animals were being moved to clean cages (82). Female rats housed alone also became significantly more active (moving, rearing, and grooming) in the initial 15 min after being present during cage change of conspecifics.

There is also evidence that being present during euthanasia and blood collection from other animals is stressful for rats. Both male and female rats showed significant elevations in HR and BP when present during decapitation of other rats, and when a paper towel with dried blood from a decapitated rat was placed atop their cage (2, 3, 82). Small but significant increases in arterial BP and HR were documented in adult male rats that were present during decapitation of other rats, especially in singly housed rats (81). Being present in the same room during decapitation and simulated decapitation of other rats also caused significant increases in HR in female rats (82).

Both plasma protein and lactate levels were significantly increased in 24 pair-housed rats decapitated within 20 sec of decapitation and blood collection of their cage-mate (83). The authors attributed the effect to latency of initial cage handling. More recent studies would suggest that the rats were also stressed by witnessing decapitation of cagemates.

Witnessing effects have not been investigated thoroughly in mice. In one study in which adult female mice, housed 5 per cage (n = 20), were successively manually restrained and decapitated, corticosterone levels exhibited two- to threefold increases in the fourth and fifth mice euthanized from each cage (4 to 8 min after the first mouse was manually restrained), suggesting a witness effect (84).

At least one study in monkeys suggests that witnessing other individuals being subjected to potentially unpleasant laboratory procedures is stressful. Macaques that were able to see other macaques being restrained and sedated for blood collection had higher cortisol levels than those that did not observe conspecific handling (85).

Discussion

Routine handling, venipuncture, and orogastric gavage lead to elevations of heart rate, blood pressure, and glucocorticoid concentrations that persist for 30 to 60 min or more following the event, suggesting that despite their routine use in laboratory studies, these procedures are acutely stressful for animals. Sharp et al. (4) noted, "Care should be exercised in dismissing a procedure as non-stressful just because it is simple or routine." Being present when these procedures are being conducted on other animals also significantly elevates physiologic parameters indicative of stress, at least in rats, mice and monkeys. Both rats and mice produce and respond to signals and odors associated with stressful laboratory procedures (86, 87).

It is reasonable to assert—as Egger et al. (88) did when they interpreted handling stress as "a psychical stress characteristic for an animal preyed upon"—that pain and fear are the primary experiential phenomena underlying the stress responses observed in the studies we reviewed. It follows, we believe, that most laboratory research on animals is intrinsically and significantly stressful, and it is therefore difficult to conclude that any studies using these procedures could be characterized as "humane" under any reasonable definition.

The complex relationship between animal handling and immune system response demonstrates evidence of both depressed and enhanced effects due to handling at both pre- and post-weaning life phases (24, 28). The studies we cite here form part of a larger body of literature that reports varying effects on immunity (e.g., tumor growth, susceptibility to infectious disease) in response to handling according to the type of tumor cell line injected, strain, age, sex, and species of the animal (26, 32). As such, it is not possible to make broad conclusions about how handling may affect the health and well-being of animals, particularly mice and rats, used in laboratory research. What is clear, however, are that handling effects can significantly alter an animal's immune status—either enhancing or compromising—and could have important methodological implications (26).

We recognize the importance of distinguishing stress from distress. Stress is commonly defined simply as the effect produced by external or internal factors and is not by itself thought to pose a threat as long as the animal can maintain an adaptive state of homeostasis. Using this benign definition, stress need not compromise animal welfare. Whether or not the reviewed procedures may lead to distress—commonly defined as the disruption of biological functions critical to the animal's well-being (89)—is for the most part not addressed in the reviewed studies. We have provided substantial evidence that routine laboratory procedures are perceived as noxious by the animals, as shown by physiologic and behavioral changes that persist for minutes or hours. This, in our view, presents both ethical and scientific problems for the traditional practice of laboratory research using animals, including the potential to complicate data interpretation.

Because pain and fear, and resulting stress and distress, may introduce confounding variability to scientific data, these states have the potential to lessen the reliability of animal studies. Scientists are well aware of this, and some have warned of the hazards of disregarding stress effects, including those arising from laboratory routines (34, 79, 90). Despite this, the negative effects of pain, stress and distress and their influence on study outcome are either not reported or underreported in published scientific papers (91).

One objection that may be made to the above conclusion is the possibility of confusing stress with arousal, or a pleasant "eustress" (92). The welfare of a mouse, for example, may be enhanced by providing an exercise wheel, yet certain physiologic parameters such as corticosterone levels, HR, and BP, may change as they would with negative stress. Sharp et al. (2) noted that it was not clear from their study whether the rats were stressed or merely aroused. Behavioral cues are helpful discriminators of stress and arousal, but few of the studies reviewed reported on animal behavior. The authors of most of the papers reviewed interpret animal stress-like responses as negative. It would seem irrational to conclude that an animal is not stressed, or is pleasantly stressed, when exhibiting pronounced physiologic changes to procedures that most animals resist and try to avoid. In the absence of evidence to the contrary, it is reasonable to conclude that stress-like responses to something that humans would find aversive (e.g., blood collection, orogastric gavage) indicate negative stress in the animal.

Where there is any question of discriminating stress from mere arousal, Principle IV of the U.S. Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training is instructive: "Unless the contrary is established, investigators should consider that procedures that cause pain or distress in human beings may cause pain or distress in other animals" (93). Blood collection and orogastric gavage both involve restraint stresses in addition to insertion of a needle or gavage tube, and Principle IV would appear to apply. In the case of animal handling, physiologic changes should be assumed to represent negative affective states in the absence of evidence to the contrary. Rats and mice, for instance, have a spontaneous fear of people, and avoid being handled unless conditioned as juveniles to accept the human hand (94, Chris Sherwin, personal communication). We conclude that stress associated with routine laboratory procedures is quantifiable and substantial.

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