Ministerie van Landbouw,
Natuur en Voedselkwaliteit

> Retouradres Postbus 20401 2500 EK Den Haag

[redacted]

Directoraat-generaal Agro en Natuur
Directie Dierlijke Agroketens en Dierenwelzijn

Bezoekadres
Bezuidenhoutseweg 73
2594 AC Den Haag

Postadres
Postbus 20401
2500 EK Den Haag

Overheidsidentificatienr
00000001003214369000

T 070 379 8911 (algemeen)
www.rijksoverheid.nl/lnv

Behandeld door
[redacted]
T 070 379 8914
[redacted]@minez.nl

Ons kenmerk
DGAN-DAD / 18003638

Uw kenmerk

Bijlage(n)
2

Datum 06 MAART 2018
Betreft Wob-besluit tenenknip

Geachte [redacted]

In uw twee brieven van 1 november 2017, allebei ontvangen op 2 november 2017, heeft u met een beroep op de Wet openbaarheid van bestuur (hierna: Wob) informatie verzocht over systematisch literatuuronderzoek tenenknip en informatie over de internationale enquête tenenknip.

Met deze brief wordt op uw twee Wob-verzoeken van 1 november 2017 besloten.

De ontvangst van uw verzoek is schriftelijk bevestigd bij brief van 15 november 2017 met kenmerk DGAN-DAD / 17177990.

In de brief van 29 november 2017 is de beslistermijn met vier weken verdaagd tot 28 december 2017.

In de brief van 12 december 2017, is aan u medegedeeld dat de beslistermijn is opgeschort tot 18 januari 2018 vanwege het vragen van zienswijzen aan derden.

In de e-mail van 16 januari 2018 is aan u toegelicht dat de afronding van het besluit op uw verzoek meer tijd vraagt dan verwacht en dat u zo spoedig mogelijk antwoord ontvangt op uw verzoek.

Wettelijk kader
Uw verzoek valt onder de reikwijdte van de Wob. Voor de relevante Wob-artikelen verwijs ik u naar de bijlage 1.

Inventarisatie documenten
Op basis van uw verzoek zijn in totaal 81 documenten inclusief bijlagen aangetroffen. Deze documenten zijn opgenomen in een inventarislijst, die als bijlage 2 bij dit besluit is gevoegd. In dit besluit wordt verwezen naar de corresponderende nummers uit de inventarislijst, zodat per document duidelijk is wat is besloten.

De documenten met nummers 3b, 4a, 5a, 5b, 6, 15a, 16a, 35b, 35c en 53a zijn reeds openbaar. De Wob is niet van toepassing op reeds openbare documenten. De vindplaats van deze documenten staat aangegeven op de inventarislijst.

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Als service stuur ik u de documenten 3b, 4a, 5a, 5b, 16a, 35c en 53a in kopie aan u toe.

De documenten met nummers 11, 12a, 13a, 13b, 13c, 25a, 31a, 42, 52a en 52b bevatten delen met informatie die buiten de reikwijdte van uw verzoek vallen. Het document met nummer 52c valt volledig buiten de reikwijdte van uw verzoek.

Derhalve is deze informatie weggelaten.

Zienswijzen
U bent er over geïnformeerd in de brief van 12 december 2017 met kenmerk DGAN-DAD / 17191860 dat er derde belanghebbenden zijn bij de openbaarmaking van de documenten 1, 2, 3, 3a, 3b, 4, 5, 5c, 6, 6a, 7, 8, 9, 10, 11, 12, 12a, 13, 13a, 13b, 13c, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 24, 25, 25a, 26, 27, 28, 29, 30, 31, 31a, 32, 33, 34, 34a, 34b, 35, 35a, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 45a, 46, 46a, 47, 48, 49, 50, 51, 52, 52a, 52b, 52c en 53a en dat deze in de gelegenheid zijn gesteld hierover zienswijzen te geven. De zienswijzen van de derde belanghebbenden heb ik in mijn belangenafweging meegenomen. Zie het onderdeel 'Overwegingen' van dit besluit.

Besluit
Ik heb besloten (deels) aan uw verzoek tegemoet te komen en de informatie waarom u verzocht, opgenomen in de documenten met nummers 1, 2, 3, 3a, 4, 5, 6a, 7, 8, 9, 10, 11, 12, 12a, 13, 13a, 13c, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 24a, 25, 25a, 26, 27, 28, 29, 30, 31, 31a, 32, 33, 34, 34a, 34b, 35, 35a, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 52a, 52b en 53 (gedeeltelijk) openbaar te maken. Voor de motivering verwijs ik naar onderdeel 'Overwegingen' van dit besluit.

Ik heb besloten de door u gevraagde informatie opgenomen in het document met nummer 5c niet openbaar te maken. Voor de motivering verwijs ik naar onderdeel Overwegingen van dit besluit.

Overwegingen
Allereerst wil ik u wijzen op het volgende. Ingevolge artikel 3, vijfde lid, van de Wob, wordt een verzoek om informatie ingewilligd met inachtneming van het bepaalde in de artikelen 10 en 11.

Het recht op openbaarmaking op grond van de Wob dient uitsluitend het publieke belang van een goede en democratische bestuursvoering. Het komt iedere burger in gelijke mate toe. Daarom kan ten aanzien van de openbaarheid geen onderscheid worden gemaakt naar gelang de persoon of de bedoeling of belangen van de verzoeker. Bij de te verrichten belangenafweging worden dan ook betrokken het algemene belang bij openbaarmaking van de gevraagde informatie en de door de weigeringsgronden te beschermen belangen, maar niet het specifieke belang van de verzoeker.

Evenmin kent de Wob een beperkte vorm van openbaarmaking. Dit betekent dat openbaarmaking van de gevraagde documenten uitsluitend aan u op grond van de Wob niet mogelijk is. Indien ik aan u de betreffende documenten verstrek, moet ik deze ook aan anderen geven indien zij daarom verzoeken. In dat licht vinden de onderstaande belangenafwegingen dan ook plaats.

Directoraat-generaal Agro en Natuur
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Ons kenmerk
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De eerbiediging van de persoonlijke levenssfeer
Op grond van artikel 10, tweede lid, aanhef en onder e, van de Wob blijft verstrekking van informatie achterwege voor zover het belang daarvan niet opweegt tegen het belang dat de persoonlijke levenssfeer wordt geëerbiedigd.

In de documenten met nummers, 1, 2, 3, 4, 5, 5c, 6a, 7, 8, 9, 10, 11, 12, 12a, 13, 13a, 13c, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 24a, 25, 26, 27, 28, 29, 30, 31, 31a, 32, 33, 34, 34b, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 52a, 52b en 53 staan persoonsgegevens, zoals namen, telefoonnummers en e-mailadressen. Ik ben van oordeel dat ten aanzien van deze gegevens het belang dat de persoonlijke levenssfeer wordt geëerbiedigd, zwaarder moet wegen dan het belang van openbaarheid. Daarom heb ik de persoonsgegevens verwijderd uit deze documenten.

Voor zover het de namen van ambtenaren betreft is hierbij het volgende van belang. Weliswaar kan, waar het gaat om beroepshalve functioneren van ambtenaren, slechts in beperkte mate een beroep worden gedaan op het belang van eerbiediging van hun persoonlijke levenssfeer. Dit ligt anders indien het betreft het openbaar maken van namen van ambtenaren. Namen zijn immers persoonsgegevens en het belang van eerbiediging van de persoonlijke levenssfeer kan zich tegen het openbaar maken daarvan verzetten. Daarbij is van belang dat het hier niet gaat om het opgeven van een naam aan een individuele burger die met een ambtenaar in contact treedt, maar om openbaarmaking van de naam in de zin van de Wob.

Persoonlijke beleidsopvattingen in een stuk voor intern beraad
Artikel 11, eerste lid, van de Wob bepaalt dat in geval van een verzoek om informatie uit documenten, opgesteld ten behoeve van intern beraad, geen informatie wordt verstrekt over daarin opgenomen persoonlijke beleidsopvattingen.

Uit de wetsgeschiedenis blijkt dat onder het begrip "documenten opgesteld ten behoeve van intern beraad" onder meer moeten worden begrepen: nota's van ambtenaren en hun politieke en ambtelijk leidinggevenden, correspondentie tussen de onderdelen van een ministerie en tussen ministeries onderling, concepten van stukken, agenda's, notulen, samenvattingen en conclusies van interne besprekingen en rapporten van ambtelijke adviescommissies. Ten aanzien van deze stukken moet van de bedoeling om ze als stukken voor intern beraad beschouwd te zien, uitdrukkelijk blijken of men moet deze bedoeling redelijkerwijs kunnen vermoeden. Deze beperking op de informatieverplichting is in de Wob opgenomen omdat een ongehinderde bijdrage van ambtenaren en van hen die van buiten bij het intern beraad zijn betrokken bij de beleidsvorming en -voorbereiding gewaarborgd moet zijn. Zij moeten in alle openhartigheid onderling en met bewindspersonen kunnen communiceren. Staatsrechtelijk zijn slechts de standpunten die het bestuursorgaan voor zijn rekening wil nemen relevant. Onder persoonlijke beleidsopvattingen worden verstaan: meningen, opinies, commentaren, voorstellen, conclusies met de daartoe aangevoerde argumenten.

De documenten met nummers 1, 2, 3, 5c, 8, 10, 11, 12, 12a, 13a, 15, 19, 20, 21, 27, 28, 29, 30, 31, 33, 34, 35, 37, 39, 40, 42, 43, 44, 45a, 46a, 48, 49, 51a, 52a, 52b en 53 zijn opgesteld ten behoeve van intern beraad en bevatten persoonlijke beleidsopvattingen. Ik verstrek daarover geen informatie.

De Afdeling Bestuursrechtspraak van de Raad van State heeft in haar uitspraak van 21 juli 2010, ECLI:NL:RVS:2010:BN1927, in rechtsoverweging 2.4.2 met een beroep op de wetsgeschiedenis concepten van stukken als stukken voor intern beraad aangemerkt, teneinde te bewerkstelligen dat bij de primaire vormgeving van het beleid betrokkenen in alle vrijheid hun gedachten en opvattingen kunnen uiten. Tevens verwijs ik naar de uitspraak van de Afdeling van 1 september 2010, ECLI:NL:RVS:2010:BN5701. Daarbij was sprake van conceptversies van antwoorden die niet openbaar zijn gemaakt, waarvan de definitieve versies wel openbaar zijn gemaakt. In r.o. 2.6.2 van die uitspraak heeft de Afdeling overwogen dat waar de conceptantwoorden afwijken van de definitieve antwoorden openbaarmaking op van grond van artikel 11 van de Wob achterwege mocht blijven. Naar mijn oordeel gaat deze redenering ook op voor de interne discussies in het onderhavige conceptdocument. Van deze documenten is de definitieve versie reeds openbaar gemaakt met uitzondering van gegevens waarop een uitzonderingsgrond van toepassing is. Op de inventarislijst en onder het kopje 'reeds openbare documenten' wordt verwezen naar de vindplaats van de definitieve documenten. Waar de feitenvaststellingen in het concept afwijken van de definitieve versie is sprake van persoonlijke beleidsopvattingen en waar zij overeenkomen met die in de definitieve versie zijn zij inmiddels openbaar en is de Wob daarop dus niet meer van toepassing.

Ik acht het niet in het belang van een goede en democratische bestuursvoering indien de standpunten van ambtenaren zelfstandig worden betrokken in de publieke discussie. Ik zie dan ook geen aanleiding om met toepassing van artikel 11, tweede lid, van de Wob in niet tot personen herleidbare vorm informatie te verstrekken over deze persoonlijke beleidsopvattingen.

Deze persoonlijke beleidsopvattingen heb ik uit de documenten verwijderd.

Wijze van openbaarmaking
De documenten die (gedeeltelijk) openbaar worden gemaakt, treft u bij dit besluit in kopie aan.

Dit besluit en de stukken die met dit besluit voor een ieder openbaar worden, worden geanonimiseerd op *www.rijksoverheid.nl* geplaatst.

Een afschrift van dit besluit zend ik aan belanghebbenden.

Directoraat-generaal Agro en Natuur
Directie Dierlijke Agroketens en Dierenwelzijn

Ons kenmerk
DGAN-DAD / 18003638

Hoogachtend,

De Minister van Landbouw, Natuur en Voedselkwaliteit,
namens deze:

[redacted]

mr. H.J.I.M. de Rooij
Plv. secretaris-generaal

[redacted]

Een belanghebbende die bezwaar heeft tegen de weigering om informatie openbaar te maken kan binnen zes weken na de dag waarop dit is bekend gemaakt een bezwaarschrift indienen. Het bezwaarschrift moet door de indiener zijn ondertekend en bevat ten minste zijn naam en adres, de dagtekening, een omschrijving van het besluit waartegen het bezwaar is gericht en de gronden waarop het bezwaar rust. Dit bezwaarschrift moet worden gericht aan: de Minister van Landbouw, Natuur en Voedselkwaliteit, directie Wetgeving en Juridische Zaken, Postbus 20401, 2500 EK 's-Gravenhage. Dit besluit is verzonden op de in de aanhef vermelde datum.

Directoraat-generaal Agro en Natuur
Directie Dierlijke Agroketens en Dierenwelzijn

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Bijlage 1 – Relevante artikelen uit de Wob

Artikel 1

In deze wet en de daarop berustende bepalingen wordt verstaan onder:

a. document: een bij een bestuursorgaan berustend schriftelijk stuk of ander materiaal dat gegevens bevat;
b. bestuurlijke aangelegenheid: een aangelegenheid die betrekking heeft op beleid van een bestuursorgaan, daaronder begrepen de voorbereiding en de uitvoering ervan;
c. intern beraad: het beraad over een bestuurlijke aangelegenheid binnen een bestuursorgaan, dan wel binnen een kring van bestuursorganen in het kader van de gezamenlijke verantwoordelijkheid voor een bestuurlijke aangelegenheid;
d. niet-ambtelijke adviescommissie: een van overheidswege ingestelde instantie, met als taak het adviseren van een of meer bestuursorganen en waarvan geen ambtenaren lid zijn, die het bestuursorgaan waaronder zij ressorteren adviseren over de onderwerpen die aan de instantie zijn voorgelegd. Ambtenaren, die secretaris of adviserend lid zijn van een adviesinstantie, worden voor de toepassing van deze bepaling niet als leden daarvan beschouwd;
e. ambtelijke of gemengd samengestelde adviescommissie: een instantie, met als taak het adviseren van één of meer bestuursorganen, die geheel of gedeeltelijk is samengesteld uit ambtenaren, tot wier functie behoort het adviseren van het bestuursorgaan waaronder zij ressorteren over de onderwerpen die aan de instantie zijn voorgelegd;
f. persoonlijke beleidsopvatting: een opvatting, voorstel, aanbeveling of conclusie van een of meer personen over een bestuurlijke aangelegenheid en de daartoe door hen aangevoerde argumenten;
g. milieu-informatie: hetgeen daaronder wordt verstaan in artikel 19.1a van de Wet milieubeheer;
h. hergebruik: het gebruik van informatie die openbaar is op grond van deze of een andere wet en die is neergelegd in documenten berustend bij een overheidsorgaan, voor andere doeleinden dan het oorspronkelijke doel binnen de publieke taak waarvoor de informatie is geproduceerd;
i. overheidsorgaan:
   1°. een orgaan van een rechtspersoon die krachtens publiekrecht is ingesteld, of
   2°. een ander persoon of college, met enig openbaar gezag bekleed.

Artikel 3

1. Een ieder kan een verzoek om informatie neergelegd in documenten over een bestuurlijke aangelegenheid richten tot een bestuursorgaan of een onder verantwoordelijkheid van een bestuursorgaan werkzame instelling, dienst of bedrijf.
2. De verzoeker vermeldt bij zijn verzoek de bestuurlijke aangelegenheid of het daarop betrekking hebbend document, waarover hij informatie wenst te ontvangen.

3. De verzoeker behoeft bij zijn verzoek geen belang te stellen.
4. Indien een verzoek te algemeen geformuleerd is, verzoekt het bestuursorgaan de verzoeker zo spoedig mogelijk om zijn verzoek te preciseren en is het hem daarbij behulpzaam.
5. Een verzoek om informatie wordt ingewilligd met inachtneming van het bepaalde in de artikelen 10 en 11.

Artikel 6
1. Het bestuursorgaan beslist op het verzoek om informatie zo spoedig mogelijk, doch uiterlijk binnen vier weken gerekend vanaf de dag na die waarop het verzoek is ontvangen.
2. Het bestuursorgaan kan de beslissing voor ten hoogste vier weken verdagen. Van de verdaging wordt voor de afloop van de eerste termijn schriftelijk gemotiveerd mededeling gedaan aan de verzoeker.
3. Onverminderd artikel 4:15 van de Algemene wet bestuursrecht wordt de termijn voor het geven van een beschikking opgeschort gerekend vanaf de dag na die waarop het bestuursorgaan de verzoeker meedeelt dat toepassing is gegeven aan artikel 4:8 van de Algemene wet bestuursrecht, tot de dag waarop door de belanghebbende of belanghebbenden een zienswijze naar voren is gebracht of de daarvoor gestelde termijn ongebruikt is verstreken.
4. Indien de opschorting, bedoeld in het derde lid, eindigt, doet het bestuursorgaan daarvan zo spoedig mogelijk mededeling aan de verzoeker, onder vermelding van de termijn binnen welke de beschikking alsnog moet worden gegeven.
5. Indien het bestuursorgaan heeft besloten informatie te verstrekken, wordt de informatie verstrekt tegelijk met de bekendmaking van het besluit, tenzij naar verwachting een belanghebbende bezwaar daar tegen heeft, in welk geval de informatie niet eerder wordt verstrekt dan twee weken nadat de beslissing is bekendgemaakt.
6. Voor zover het verzoek betrekking heeft op het verstrekken van milieu-informatie:
   a. bedraagt de uiterste beslistermijn in afwijking van het eerste lid twee weken indien het bestuursorgaan voornemens is de milieu-informatie te verstrekken terwijl naar verwachting een belanghebbende daar bezwaar tegen heeft;
   b. kan de beslissing slechts worden verdaagd op grond van het tweede lid, indien de omvang of de gecompliceerdheid van de milieu-informatie een verlenging rechtvaardigt;
   c. zijn het derde en vierde lid niet van toepassing.

Artikel 10
1. Het verstrekken van informatie ingevolge deze wet blijft achterwege voor zover dit:
   a. de eenheid van de Kroon in gevaar zou kunnen brengen;
   b. de veiligheid van de Staat zou kunnen schaden;
   c. bedrijfs- en fabricagegegevens betreft, die door natuurlijke personen of rechtspersonen vertrouwelijk aan de overheid zijn meegedeeld;

d. persoonsgegevens betreft als bedoeld in paragraaf 2 van hoofdstuk 2 van de Wet bescherming persoonsgegevens, tenzij de verstrekking kennelijk geen inbreuk op de persoonlijke levenssfeer maakt.

2. Het verstrekken van informatie ingevolge deze wet blijft eveneens achterwege voor zover het belang daarvan niet opweegt tegen de volgende belangen:
   a. de betrekkingen van Nederland met andere staten en met internationale organisaties;
   b. de economische of financiële belangen van de Staat, de andere publiekrechtelijke lichamen of de in artikel 1a, onder c en d, bedoelde bestuursorganen;
   c. de opsporing en vervolging van strafbare feiten;
   d. inspectie, controle en toezicht door bestuursorganen;
   e. de eerbiediging van de persoonlijke levenssfeer;
   f. het belang, dat de geadresseerde erbij heeft als eerste kennis te kunnen nemen van de informatie;
   g. het voorkomen van onevenredige bevoordeling of benadeling van bij de aangelegenheid betrokken natuurlijke personen of rechtspersonen dan wel van derden.
3. Het tweede lid, aanhef en onder e, is niet van toepassing voorzover de betrokken persoon heeft ingestemd met openbaarmaking.
4. Het eerste lid, aanhef en onder c en d, het tweede lid, aanhef en onder e, en het zevende lid, aanhef en onder a, zijn niet van toepassing voorzover het milieu-informatie betreft die betrekking heeft op emissies in het milieu. Voorts blijft in afwijking van het eerste lid, aanhef en onder c, het verstrekken van milieu-informatie uitsluitend achterwege voorzover het belang van openbaarmaking niet opweegt tegen het daar genoemde belang.
5. Het tweede lid, aanhef en onder b, is van toepassing op het verstrekken van milieu-informatie voor zover deze handelingen betreft met een vertrouwelijk karakter.
6. Het tweede lid, aanhef en onder g, is niet van toepassing op het verstrekken van milieu-informatie.
7. Het verstrekken van milieu-informatie ingevolge deze wet blijft eveneens achterwege voorzover het belang daarvan niet opweegt tegen de volgende belangen:
   a. de bescherming van het milieu waarop deze informatie betrekking heeft;
   b. de beveiliging van bedrijven en het voorkomen van sabotage.
8. Voorzover het vierde lid, eerste volzin, niet van toepassing is, wordt bij het toepassen van het eerste, tweede en zevende lid op milieu-informatie in aanmerking genomen of deze informatie betrekking heeft op emissies in het milieu.

Artikel 11

1. In geval van een verzoek om informatie uit documenten, opgesteld ten behoeve van intern beraad, wordt geen informatie verstrekt over daarin opgenomen persoonlijke beleidsopvattingen.
2. Over persoonlijke beleidsopvattingen kan met het oog op een goede en democratische bestuursvoering informatie worden verstrekt in niet tot personen

herleidbare vorm. Indien degene die deze opvattingen heeft geuit of zich erachter heeft gesteld, daarmee heeft ingestemd, kan de informatie in tot personen herleidbare vorm worden verstrekt.
3. Met betrekking tot adviezen van een ambtelijke of gemengd samengestelde adviescommissie kan het verstrekken van informatie over de daarin opgenomen persoonlijke beleidsopvattingen plaatsvinden, indien het voornemen daartoe door het bestuursorgaan dat het rechtstreeks aangaat aan de leden van de adviescommissie voor de aanvang van hun werkzaamheden kenbaar is gemaakt.
4. In afwijking van het eerste lid wordt bij milieu-informatie het belang van de bescherming van de persoonlijke beleidsopvattingen afgewogen tegen het belang van openbaarmaking. Informatie over persoonlijke beleidsopvattingen kan worden verstrekt in niet tot personen herleidbare vorm. Het tweede lid, tweede volzin, is van overeenkomstige toepassing.

Directoraat-generaal Agro en Natuur
Directie Dierlijke Agroketens en Dierenwelzijn

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Bijlage 2 - Inventarislijst

Nr.	Document	Beoordeling	Wob	Afzender	Ontvanger
1	Emailwisseling 10 november 2016	Deels Openbaar	10.2.e 11.1	EZ, VWS	VWS, EZ
2	Emailwisseling 6 december 2016	Deels Openbaar	10.2.e 11.1	EZ, ZonMw	EZ, ZonMw
3	Emailwisseling 17 maart 2016 e.v.	Deels Openbaar	10.2.e 11.1	EZ, ZonMw	EZ, ZonMw
3a	NL conceptversie van de review	Openbaar		ZonMw	EZ
3b	ENG conceptversie van de review	Reeds Openbaar https://www.ncbi.nlm.nih.gov/pubmed/28429644		ZonMw	EZ
4	Email 7 april 2016	Deels Openbaar	10.2.e	EZ	ZonMw
4a	Motie Graus	Reeds Openbaar https://zoek.officielebekendmakingen.nl/kst-32336-48.html			
5	Emailwisseling 23 juni 2016	Deels Openbaar	10.2.e	ZonMw	EZ
5a	Working Party Report	Reeds Openbaar https://www.ncbi.nlm.nih.gov/pubmed/23467487		ZonMw	EZ
5b	Rapportage	Reeds Openbaar https://norecopa.no/media/6470/norecopa-toeclip.pdf		ZonMw	EZ
5c	Concept Rapport ZonMW	Niet openbaar	10.2.e 11.1	ZonMw	EZ
6	Brief d.d. 24 juni 2016	Reeds Openbaar ttps://www.rijksoverheid.nl/documenten/brieven/2016/06/24/inventarisatie-gebruik-teenkootknip-en-oorknip		ZonMw	EZ
6a	Emailwisseling 24 juni 2016	Deels Openbaar	10.2.e	ZonMw	EZ
7	Emailwisseling 22 december 2014 e.v.	Deels Openbaar	10.2.e	ZonMw, EZ	EZ, ZonMw

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8	Emailwisseling 13 januari 2015 e.v.	Deels Openbaar	10.2.e 11.1	EZ	EZ
9	Emailwisseling 8 januari 2015	Deels Openbaar	10.2.e	EZ	ZonMw
10	Emailwisseling 13 januari 2015	Deels Openbaar	10.2.e 11.1	EZ	ZonMw
11	Emailwisseling 14 januari 2015	Deels buiten reikwijdte Deels Openbaar	10.2.e 11.1	ZonMw	EZ
12	Emailwisseling 20 januari 2015	Deels Openbaar	10.2.e 11.1	EZ	EZ
12 a	Aanvraagformulier verplichting	Deels Openbaar Deels buiten reikwijdte	10.2.e 11.1	EZ	EZ
13	Email wisseling/ Verslag ethische reflectie over de teenknip bij muizen	Deels Openbaar	10.2.e	EZ	EZ
13 a	Opdrachtbrief d.d. 30 januari 2015	Deels Openbaar Deels buiten reikwijdte	10.2.e 11.1	EZ	ZonMw
13 b	Verslag ethische reflectie teenknip	Volledig buiten reikwijdte		EZ	EZ
13 c	Brief standpunt RODA d.d. 20 november 2014	Deels buiten reikwijdte Deels Openbaar	10.2.e	RODA	EZ
14	Emailwisseling 12 februari 2015	Deels Openbaar	10.2.e	ZonMw, EZ	EZ, ZonMw
15	Emailwisseling 23 februari 2015	Deels Openbaar	10.2.e 11.1	EZ	ZonMw
15 a	Verslag AO 10 februari 2015	Reeds Openbaar https://zoek.officielebekendmakingen.nl/kst-32336-			

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Ons kenmerk
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		40.html			
16	Emailwisseling 31 maart 2015 e.v.	Deels Openbaar	10.2.e	ZonMw	EZ
16 a	ENG Review	Reeds Openbaar http://journals.sagepub.com/doi/pdf/10.1177/0023677212473918		ZonMw	EZ
17	Emailwisseling 20 april 2015.	Deels Openbaar	10.2.e	EZ	ZonMw
18	Emailwisseling 8 juni 2015	Deels Openbaar	10.2.e	ZonMw, EZ	EZ, ZonMw
19	Emailwisseling 1 juli 2015	Deels Openbaar	10.2.e 11.1	EZ	EZ
20	Emailwisseling 2 juli 2015 e.v.	Deels Openbaar	10.2.e 11.1	EZ, ZonMw	ZonMw, EZ
21	Emailwisseling 24 augustus 2015	Deels Openbaar	10.2.e 11.1	ZonMw, EZ	EZ, ZonMw
22	Emailwisseling 7 september 2015	Deels Openbaar	10.2.e	EZ, ZonMw	ZonMw, EZ
23	Emailwisseling 14 september 2015	Deels Openbaar	10.2.e	ZonMw, EZ	EZ, ZonMw
24	Emailwisseling 14 september 2015	Deels Openbaar	10.2.e	ZonMw	EZ
24 a	Conceptbrief aan Syrcle Nijmegen	Deels Openbaar	10.2.e	ZonMw	EZ
25	Emailwisseling 15 september 2015	Deels Openbaar	10.2.e	ZonMw	EZ
25 a	Voortgangsverslag	Deels buiten reikwijdte		ZonMw	EZ
26	Emailwisseling 21 september 2015	Deels Openbaar	10.2.e	EZ, ZonMw	ZonMw, EZ
27	Emailwisseling 4 oktober 2015	Deels Openbaar	10.2.e 11.1	ZonMw, EZ	EZ, ZonMw
28	Emailwisseling 3 oktober	Deels Openbaar Deels buiten reikwijdte	10.2.e 11.1	ZonMw, EZ	EZ, ZonMw

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	2015				
29	Emailwisseling 17 november 2015 e.v.	Deels Openbaar	10.2.e 11.1	VWS, EZ	EZ, VWS
30	Emailwisseling 24 november 2015 e.v.	Deels Openbaar	10.2.e 11.1	NVWA, ZonMw, EZ	EZ, ZonMw, NVWA,
31	Emailwisseling 5 januari 2016 e.v.	Deels Openbaar	10.2.e 11.1	EZ, VWS	EZ, VWS
31 a	Goedkeurings brief 2016	Deels Openbaar Deels buiten reikwijdte	10.2.e	VWS	ZonMw
32	Emailwisseling 8 februari 2016 e.v.	Deels Openbaar	10.2.e	ZonMw, EZ	EZ, ZonMw
33	Emailwisseling 1 maart 2016 e.v.	Deels Openbaar	10.2.e 11.1	EZ, ZonMw	ZonMw, EZ
34	Emailwisseling 1 maart 2016	Deels Openbaar	10.2.e 11.1	EZ, ZonMw	ZonMw, EZ
34 a	Bijlage bij mail	Openbaar		EZ, ZonMw	ZonMw, EZ
34 b	Bijlage bij mail	Deels Openbaar	10.2.e	EZ, ZonMw	ZonMw, EZ
35	Emailwisseling 8 maart 2016	Deels Openbaar	10.2.e 11.1	EZ, ZonMw	ZonMw, EZ
35 a	Bijlage	Openbaar		EZ, ZonMw	ZonMw, EZ
35 b	Bijlage	Reeds Openbaar http://www.ingentaconnect.com/content/ben/cppm/2013/00000011/00000001/art00004?crawler=true (tegen betaling)		EZ, ZonMw	ZonMw, EZ
35 c	Bijlage	Reeds Openbaar http://journals.sagepub.com/doi/pdf/10.1258/002367704195898 1		EZ, ZonMw	ZonMw, EZ
36	Emailwisseling 17 maart 2016 e.v.	Deels Openbaar	10.2.e	ZonMw	EZ
37	Emailwisseling 5 april 2016 e.v.	Deels Openbaar	10.2.e 11.1	ZonMw, EZ	EZ, ZonMw
38	Emailwisseling	Deels Openbaar	10.2.e	EZ	ZonMw

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	7 april 2016				
39	Emailwisseling 3 mei 2016 e.v.	Deels Openbaar	10.2.e 11.1	ZonMw	EZ
40	Emailwisseling 24 mei 2016 e.v.	Deels Openbaar	10.2.e 11.1	ZonMw, EZ	EZ, ZonMw
41	Emailwisseling 30 mei 2016	Deels Openbaar	10.2.e	EZ, ZonMw	EZ
42	Emailwisseling 30 mei 2016	Deels Openbaar Deels buiten reikwijdte	10.2.e 11.1	EZ	EZ
43	Emailwisseling 31 mei 2016	Deels Openbaar	10.2.e 11.1	EZ, ZonMw	ZonMw, EZ
44	Emailwisseling 14 juni 2016	Deels Openbaar	10.2.e 11.1	EZ, ZonMw	ZonMw, EZ
45	Emailwisseling 23 juni 2016	Deels Openbaar	10.2.e	ZonMw	EZ
45 a	Bijlage bij email concept versie rapport	Niet Openbaar	11.1	ZonMw	EZ
46	Emailwisseling 23 juni 2016	Deels Openbaar	10.2.e	EZ	ZonMw
46 a	Bijlage bij email Concept versie rapport	Niet openbaar	11.1	EZ	ZonMw
47	Emailwisseling 23 juni 2016	Deels Openbaar	10.2.e	EZ	EZ
48	Emailwisseling 23 juni 2016	Deels Openbaar	10.2.e 11.1	EZ	EZ
49	Emailwisseling 23 juni 2016	Deels Openbaar	10.2.e 11.1	EZ	EZ, ZonMw
50	Emailwisseling 18 juli 2016	Deels Openbaar	10.2.e	EZ	ZonMw
51	Emailwisseling 8 augustus 2016	Deels Openbaar	10.2.e	EZ	EZ
51 a	Concept Nota	Niet Openbaar	11.1	EZ	
52	Emailwisseling 20 september 2016	Deels Openbaar	10.2.e	ZonMw	EZ
52 a	Bijlage conceptagend a	Deels Openbaar Deels buiten reikwijdte	10.2.e 11.1	ZonMw	EZ

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52 b	Bijlage notulen	Deels Openbaar Deels buiten reikwijdte	10.2.e 11.1	ZonMw	EZ
52 c	Bijlage Routebeschrij-ving	Volledig buiten reikwijdte		ZonMw	EZ
53	Nota aan Stas	Deels Openbaar	10.2.e 11.1	EZ	EZ
53 a	Bijlage Reeds Openbaar rapport ZonMw	Volledig Openbaar https://www.rijksoverheid.nl /documenten/brieven/2016/ 06/24/inventarisatie-gebruik-teenkootknip-en-oorknip		EZ	

Van:
Verzonden: donderdag 16 november 2017 10:01
Aan:
Onderwerp: FW: Reactie op ZonMw jaarplan 2017 ten behoeve van goedkeuringbrief 2017

Tenenknip

Van:
Verzonden: donderdag 10 november 2016 16:25
Aan:
CC:
Onderwerp: RE: Reactie op ZonMw jaarplan 2017 ten behoeve van goedkeuringbrief 2017

Ha

Mij schiet toch nog iets te binnen. Graag de volgende tekst opnemen:

'ZonMw is en marge van het programma Meer Kennis met Minder Dieren verzocht enkele activiteiten te ontplooien op het gebied van de teenknip. Kortheidshalve wordt in dit verband verwezen naar de opdrachtbrief van 30 januari 2015, kenmerk DGA-AK/15009273. Bij schrijven d.d. 24 juni 2016, uw kenmerk 2016/13344/ZONMW inzake dossier. nr. 40-42600-20, heeft u aan het ministerie van Economische Zaken (EZ) uw rapportage aangeboden met de resultaten hiervan. Naar aanleiding van en in vervolg op voornoemde rapportage zult u verzocht worden uw activiteiten op het gebied van de teenknip voort te zetten. De inhoudelijk contactpersoon van EZ zal hierover contact met u opnemen.'

Bij voorbaat dank voor de medewerking.

Groet,

Dag

Geen nadere opmerkingen nav ZonMw-jaarplan 2017.

Gr.

Van: @minvws.nl]
Verzonden: woensdag 2 november 2016 10:41
Aan:

Onderwerp: Reactie op ZonMw jaarplan 2017 ten behoeve van goedkeuringbrief 2017

Beste allemaal,

Van ZonMw kregen we, in reactie op de algemene opdrachtbrief 2017, het jaarplan 2017 toegestuurd (zie bijlage). Voor 2017 heeft ZonMw zijn jaarplan anders opgezet dan voorheen en de programma's langs de lijnen van 14

overkoepelende clusters geordend (zie onderaan een overzicht van de clusters en de contactpersonen wiens programma(s) daarbinnen vallen).

Wil je voor het programma(s) waarvan jij de contactpersoon bent een blik werpen op de beschreven doelen, stakeholders en voorgenomen activiteiten in 2017, en mij **uiterlijk donderdag 17 november** laten weten of er bijzonderheden zijn die nog aan ZonMw meegegeven moeten worden in de goedkeuringsbrief 2017. Uitgangspunt voor je programma(s) zijn uiteraard de afspraken die voor 2017 al zijn vastgelegd in het oorspronkelijke programmavoorstel, en eventuele aanwijzingen die je voor de zomer al aan ZonMw hebt meegegeven via de algemene opdrachtbrief.

Alvast heel erg bedankt!

Vriendelijke groet,

Overzicht clusters en contactpersonen

Voorheen Wetenschap en Innovatie

- Fundamenteel onderzoek en talent p. 22 t.e.m. 24 (
- Life Sciences and Health p. 47 t.e.m. 49 (
- Translationeel onderzoek p. 64 t.e.m. 67

Voorheen Preventie

- Preventie p. 56 t.e.m. 59
- Gezondheidsbescherming p. 34 t.e.m. 37
- Sport en Bewegen p. 60 t.e.m. 63

Voorheen Kwaliteit en Doelmatigheid

- Doelmatigheidsonderzoek p. 18 t.e.m. 21
- Geneesmiddelen p. 30 t.e.m. 33
- Kwaliteit van Zorg p. 41 t.e.m. 45
- Palliatieve Zorg p. 53 t.e.m. 55

Voorheen Zorg en Welzijn

- GGZ p. 25 t.e.m. 27
- Gehandicapten en Chronisch Zieken p. 28 t.e.m. 29
- Jeugd p. 38 t.e.m. 40 (
- Ouderen p. 50 t.e.m. 52

Overige programma's die niet in een cluster vallen

- Topzorg p. 46
- Forum biotechnologie en genetica p. 68
- Evaluatie regelgeving p. 69
- Ethiek en gezondheid p. 70
- Gender en Gezondheid p. 71

Implementatie advies Denktank Aanvullende financiering alternatieven voor dierproeven (denktank)

-
-
-
-

ZonMw

-

Van:
Verzonden: donderdag 16 november 2017 10:03
Aan:
Onderwerp: FW: SPOED!! Onder embargo teksten uit voortgangsrapportage aan TK

Urgentie: Hoog

tenenknip

Van:
Verzonden: dinsdag 6 december 2016 13:34
Aan:
CC:
Onderwerp: FW: SPOED!! Onder embargo teksten uit voortgangsrapportage aan TK
Urgentie: Hoog

Reactie op passages uit TK brief. Zie onderaan de teenknip. Ben je akkoord met het schrappen van 'samen met experts' etc.?

. Lijkt me reeel dat dat nog een punt is waar naar gekeken moet worden.

Graag je reactie. Dank.

Van: @zonmw.nl]
Verzonden: dinsdag 6 december 2016 13:24
Aan:
CC:
Onderwerp: FW: SPOED!! Onder embargo teksten uit voortgangsrapportage aan TK
Urgentie: Hoog

Hoi

Hierbij mijn reactie.

Ik ben hierna niet meer bereikbaar omdat ik onderweg ben naar Londen.
Ik ben maandag 12 december weer terug.

Gr

Van: @minez.nl]
Verzonden: dinsdag 6 december 2016 11:05
Aan: @zonmw.nl>
CC: @zonmw.nl>; @minez.nl>
Onderwerp: SPOED!! Onder embargo teksten uit voortgangsrapportage aan TK
Urgentie: Hoog

Beste

Onder embargo t/m 15 december a.s. (datum verzending voortgangsrapportage Plan van aanpak Dierproeven en alternatieven naar de TK) leg ik jullie de onderhavige teksten uit de voortgangsbrief aan de TK voor met de mogelijkheid correcties hierin aan te brengen indien ze naar jullie mening feitelijk niet juist zijn:

Van:)
Verzonden: donderdag 16 november 2017 10:11
Aan:)
Onderwerp: FW: SR teenknip en oorknip
Bijlagen: NL vertaling teenknip_evo_KW.docx; manuscript SR toe and earclip_EZ_evo_KW.docx

Van:
Verzonden: donderdag 17 maart 2016 9:24
Aan:
Onderwerp: FW: SR teenknip en oorknip

, Lees je even mee? Graag ook jouw opinie. Dank. Gr.

Van: @zonmw.nl]
Verzonden: dinsdag 15 maart 2016 14:57
Aan:
CC:
Onderwerp: SR teenknip en oorknip

Hoi ,

Bij deze stuur ik je de aangepaste versies van het Engelstalige artikel van de systematic review over de teenknip en oorknip.
En de Nederlandstalige samenvatting met daarin de vragen uit de opdrachtbrief apart behandeld.
ik hoop dat jij je daar ook in kan vinden.

We spreken elkaar in ieder geval as maandag.

Met vriendelijke groet,

Telefoon:
Aanwezig op: ma, di, wo, do
@zonmw.nl

ZonMw
Laan van Nieuw Oost Indië 334, 2593 CE Den Haag
Postbus 93245, 2509 AE Den Haag
www.zonmw.nl

[redacted] I ~~Dat~~ houdt in dat de industrie om vraagstukken/onderzoeksproblemen (challenges) zal worden gevraagd. Kennisinstellingen kunnen vervolgens op een dergelijke probleemstelling inschrijven als ze denken een oplossing daarvoor te kunnen leveren. [redacted]

Teenknip
In de tweede voortgangsrapportage d.d. 1 november 2015 heb ik u uitvoerig geschetst welke activiteiten ZonMw ondernam op het gebied van de teenknip. ZonMw heeft mij inmiddels haar rapport inzake de teenknip aangeboden, dat u eveneens in bijlage aantreft (bijlage 4). Hiermee doe ik mijn toezegging in het Algemeen overleg Dierproeven d.d. 2 maart jl. om u te informeren gestand.

ZonMw geeft aan dat over een gemiddelde van twee jaar en met in acht name van veel ontbrekende en geschatte informatie de ruwe schatting met betrekking tot de omvang van de toepassing van de teenknip is dat deze jaarlijks bij tussen de 134.000 en 167.000 dieren wordt toegepast.
De enquête onder (inter)nationale experts had – ondanks herhaalde oproepen – slechts een respons van 10 % en leverde onvoldoende aanknopingspunten voor identificatie van kansrijke alternatieven voor de teenknip voor het opzetten van een module binnen 'Meer Kennis met Minder Dieren'.
ZonMw heeft wel veel methoden kunnen identificeren die geschikt zijn voor óf genotypering óf identificatie van proefdieren. Maar er is nog steeds géén beter alternatief voor genotypering en identificatie op jonge leeftijd via de uitvoering van één handeling bekend dan de teenknip. De 'synthesis of evidence' uitgevoerd door het Systematic Review Centre for Laboratory anmial Experimentation (SYRCLE) van het UMC Radboud te Nijmegen, die tot doel had wetenschappelijke onderbouwing te krijgen voor de mate van ongerief van de teenknip en de oorknip heeft helaas niet tot het gewenste inzicht geleid. SYRCLE heeft gesteld dat - gebaseerd op het huidige bewijs - een effect van de teen(koot)knip of van de oorknip op het dierenwelzijn noch kan worden uitgesloten noch kan worden bevestigd.

Juist omdat onduidelijkheid bestaat over de mate van ongerief veroorzaakt door de teenknip en het ontbreken van een volwaardig alternatieve methode, wil ik uit voorzorg de toepassing van deze ingreep zoveel mogelijk beperken. Ik heb ZonMw inmiddels gevraagd om [redacted]
Diergeneeskundigen (zo weinig mogelijk ingrepen, voldoen aan het zorgvuldigheidsvereiste, zo verantwoord mogelijk de ingrepen toepassen). Hiermee wil ik de toepassing van de ingreep geëigender en nog zorgvuldiger maken. De teenknip mag alleen worden uitgevoerd voor een zeer beperkt aantal doelstellingen en dient op een zo zorgvuldig mogelijke manier te worden uitgevoerd. De mogelijke testsituaties met de meest geëigende ingreep in die context worden (opnieuw) beschreven; hierbij kan worden voortgeborduurd op de bestaande richtlijn van de 'Federation of Laboratory Anial Science Association (FELASA). De NVWA zal de naleving van deze Code of best practice handhaven bij de inspecties.

<u>Bij deze is dus ook het verzoek aan jullie gedaan om de activiteiten rond de teenknip voort te zetten zoals hiervoor omschreven.</u>
[redacted]

Graag je reactie. Alvast bedankt.

Groet,
[redacted]

3a

Een systematisch review naar ongerief veroorzaakt door teenknip of oorknip in knaagdieren.

Kimberley E Wever[1], Florentine J Geessink[1], Michelle Brouwer[1], Alice Tillema[2] and Merel Ritskes-Hoitinga[1]

[1]SYstematic Review Centre for Laboratory animal Experimentation (SYRCLE) and [2]Medical Library, Radboud university medical center, Nijmegen, The Netherlands

Samenvatting

Betrouwbare identificatie van afzonderlijke proefdieren is vereiste in veel dierproeven. Identificatiemethoden moeten doeltreffend en praktisch zijn, en daarnaast zo min mogelijk ongerief voor het dier te weeg brengen, zodat er geen ongewenste variabiliteit in het experiment optreedt. De teenknip en oorknip worden vaak gebruikt voor de individuele identificatie en genotypering van muizen en ratten. Er wordt verondersteld dat deze ingrepen negatieve effecten op het dierenwelzijn hebben, echter, tot nu toe is het beschikbare wetenschappelijk bewijs over dit onderwerp nog niet eerder middels een systematisch review onderzocht. We voerden een systematische review uit van het bewijs voor het ongerief als gevolg van de teenknip en oorknip, om de dierexperimenteel onderzoekers en andere belanghebbenden die betrokken zijn bij de keuze van de identificatiemethoden voor knaagdieren beter te informeren.

Door middel van een systematische zoekstrategie in drie databases identificeerden we zeven studies naar het effect van de oorknip en vijf studies naar het effect van de teenknip op dierenwelzijn. Twee van deze studies zijn uitgevoerd in ratten, de anderen in muizen. De studiekarakteristieken waren zeer heterogeen in termen van leeftijd, ras en geslacht van de dieren, de timing van de interventie en het aantal uitgevoerde teen- of oorkippen. Onze analyse van het risico op bias toonde aan dat de rapportage van de studiemethoden en maatregelen om bias te voorkomen ontoereikend was, wat leidt tot een onduidelijk of hoog risico op bias in alle studies. Geen van de studies rapporteerde een powerberekening om de gekozen steekproefomvang te rechtvaardigen. Daarnaast is een zeer groot aantal uitkomstmaten met betrekking tot het welzijn van dieren gerapporteerd (bijna vijftig verschillende uitkomstmaten in teenknip studies en vijftien verschillende uitkomsten in oorknip studies). Tussen al deze uitkomstmaten vonden we bewijs voor ongerief als gevolg van de oorknip in de vorm van een toename in ademvolume, vocalisatie (piepen) en bloeddruk. Voor de teenknip werd in pups een toename in vocalisatie en een verminderde motorische activiteit gevonden, en op lange termijn een afname in grijpkracht en zwemvermogen in volwassen dieren.

We concluderen dat er te weinig bewijs is om het ongerief als gevolg van de teenknip of oorknip betrouwbaar te kunnen vaststellen en dat de kwaliteit van het beschikbare bewijsmateriaal onduidelijk is. Adequaat onderbouwde, kwalitatief hoogwaardige studies met betrouwbare, relevante uitkomstmaten zijn nodig om de impact van deze identificatiemethoden nauwkeurig te kunnen beoordelen. Totdat er meer betrouwbare gegevens beschikbaar zijn kan een effect van teenknip of oorknip op het dierenwelzijn en de onderzoeksresultaten niet worden uitgesloten of bevestigd.

Beantwoording hoofdvraag en deelvragen van dit review

- *Hoofdvraag: wat is, op basis van het beschikbare bewijs, het ongerief ten gevolge van de teenknip en de oorknip bij proefdieren?*

De meeste uitkomstmaten toonden geen effect van teen- of oorknip op ongerief aan. Aan de andere kant zijn er ook uitkomstmaten die wel bewijs voor ongerief aantonen, voor zowel de oorknip (verhoogd ademvolume, vocalisatie en bloeddruk) als voor de teenknip (meer vocalisatie, minder activiteit in pups en minder grijpkracht en zwemvermogen in volwassenen). Totdat er meer betrouwbare gegevens beschikbaar zijn kan een effect van teenknip of oorknip op het dierenwelzijn en de onderzoeksresultaten niet worden uitgesloten of bevestigd.

- *Deelvraag 1: welk bewijs is er beschikbaar met betrekking tot het ongerief ten gevolge van de teenknip en de oorknip?*

Studies die onderzoek doen naar de effecten van de teen- en oorknip op het welzijn van knaagdieren zijn schaars en erg heterogeen. Deze heterogeniteit wordt voornamelijk veroorzaakt door verschillen in de populatie (geslacht, stam) en de interventie (leeftijd op moment van knippen, aantal plaatsen geknipt), evenals het grote aantal verschillende uitkomstmaten dat gemeten is.

- *Deelvraag 2: Wat is de kwaliteit van dit bewijs?*

De kwaliteit is laag en meerdere beperkingen van de primaire studies verminderen de betrouwbaarheid van zowel het bewijs vóór als tegen een effect op ongerief. Onze kwaliteitsbeoordeling laat zien dat de rapportage van de dierstudies ontoereikend is. Daardoor werd het risico op de meeste vormen van bias als onduidelijk gescoord en is er een onduidelijk tot groot risico dat onderzoeksresultaten door bias zijn verstoord. Het specificeren van een primaire uitkomstmaat voorkomt het aanpassen van de uitkomstmaat op basis van de studieresultaten en daarmee het risico op bias. Echter, geen van de studies definieerde welke uitkomstmaat de primaire uitkomstmaat was. Ook werd er in geen van de studies een power berekening gerapporteerd, terwijl een onderbouwing van het aantal dieren per groep door veel ethische commissies verplicht wordt gesteld. Op dit moment is het onmogelijk om te bepalen of de steekproefgrootte in een van de studies toereikend was om een verschil tussen te experimentele groepen aan te tonen. De gebruikte controlegroepen waren soms niet (optimaal) geschikt om het effect van teen- of oorknip op ongerief aan te kunnen tonen. Daarnaast tonen we aan dat de data van verschillende uitkomstmaten onvolledig zijn gerapporteerd.

- *Deelvraag 3: welke factoren beïnvloeden het ongerief ten gevolge van de teenknip en de oorknip?*

Het bestaande bewijs is te beperkt en te heterogeen om betrouwbaar de invloed van bijvoorbeeld diersoort, leeftijd, geslacht, ras, of de methode van knippen op de ernst van het ongerief te beoordelen. Drie studies deden afzonderlijke analyses voor mannen en vrouwen, maar de effecten verschilden niet tussen de verschillende sexen.

- *Deelvraag 4: hoe verhouden de teenknip en de oorknip zich tot elkaar met betrekking tot het veroorzaakte ongerief?*

Het bestaande bewijs is te beperkt en te heterogeen om een betrouwbare vergelijking te kunnen maken tussen de teen- en oorknip. De gerapporteerde uitkomstmaten zijn erg verschillend tussen de teen- en oorknip, en daardoor niet te vergelijken. Het is moeilijk om een uitkomstmaat te vinden die bij zowel teen- als oorknippen op het moment van knippen gemeten kan worden, omdat de teenknip over het algemeen niet wordt uitgevoerd in pups ouder dan 7 dagen, terwijl oorknip pas na 14 dagen en vaak nog veel later wordt toegepast.

- *Deelvraag 5: welke leeftijd van het proefdier, techniek, plaats van ingreep geeft voor de teenknip en oorknip de minste vorm van pijn en ongerief?*

Ook hier is het bestaande bewijs te beperkt en te heterogeen om een betrouwbare uitspraak te doen over de optimale leeftijd of techniek van de ingreep. Schaeffer et al. vonden dat 3 dagen oude pups die een teenknip ondergingen een lagere grijpkracht hadden dan 7 dagen oude pups. Dit werd toegeschreven aan het feit dat de tenen op dag 3 nog deels vergroeid zijn en te klein zijn om nauwkeurig te knippen, waardoor er een te groot deel van de teen verwijderd wordt. Op basis hiervan zou het knippen van de teen voor dag 3 niet aan te raden zijn, maar replicatie van dit onderzoek is nodig om dit te bevestigen. Studies waarin de teenknip uitgevoerd werd op dag 4 en dag 5 rapporteerden dat de procedure snel en eenvoudig toe te passen was, terwijl onze heranalyse van de data van Paluch et al. suggereert dat er ongerief optreed in zeven dagen oude pups (toename vocalisatie, verminderde motorische activiteit), maar niet in zeventien dagen oude pups. Hieruit

concluderen we dat het effect van leeftijd onduidelijk is en dat er verder onderzoek op dit gebied nodig is.
Eén studie onderzocht het toepassen van pijnstillende vapocoolant spray tijdens de teenknip, maar rapporteerde dat deze spray tenen aan elkaar plakte waardoor het risico op onjuist knippen toenam en er een toename was van ongerief en stress door het langduriger hanteren van de dieren. Ook verstoorde de spray de bloedstolling na het knippen. Op basis hiervan is het toepassen van deze pijnstillende spray niet te adviseren, maar verder onderzoek naar mogelijke andere pijnstillers of (lokale) anesthetica kunnen de moeite waard zijn.

Introductie
Knaagdieren, met name muizen en ratten, zijn de meest gebruikte proefdieren in biomedisch onderzoek. Ze zijn meestal identiek qua uiterlijk en gehuisvest in groepen. Individuele identificatie van de dieren is vaak noodzakelijk tijdens de fokkerij, de dagelijkse zorg of experimentele procedures. Een aantal identificatiemethoden zijn hiervoor in gebruik. Selectie van de optimale methode voor individuele identificatie is afhankelijk van verschillende factoren, waaronder diersoort, leeftijd, huidpigmentatie, onderzoeksduur en de beschikbare technische deskundigheid. De ideale identificatiemethode is doeltreffend en doelmatig, maar geeft ook zo min mogelijk pijn en / of ongerief voor het dier, omdat dit kan interfereren met het welzijn van de dieren en als gevolg daarvan de experimentele resultaten kan verstoren. Het is daarom belangrijk om het effect van identificatiemethoden op het dierenwelzijn te beoordelen.
De teenknip is een individuele identificatiemethode, meestal gebruikt in muizen, die in pasgeboren en zeer jonge dieren kan worden toegepast. Het meest distale deel van de tweede falanx, of een groter deel van de teen, wordt verwijderd. Het verwijderde weefsel kan worden gebruikt voor genotypering. De oorknip wordt gebruikt om volwassen knaagdieren (muizen en ratten) te identificeren. Met een speciale perforator worden gaten of inkepingen in het oor geknipt, volgens een schema corresponderend met een nummertabel. Ook hier kan het weggeknipte weefsel worden gebruikt voor genotypering.
De ethische rechtvaardiging om deze methoden uit te voeren is een onderwerp van debat, omdat beide methodes waarschijnlijk pijn en / of ongerief veroorzaken. Beide methoden vereisen het fixeren van de dieren en kunnen het welzijn van de dieren permanent beïnvloeden. Zo kan de teenknip het vermogen om te lopen, grijpen, wassen en voeden aantasten. Echter, het in de literatuur beschikbare wetenschappelijk bewijs voor ongerief veroorzaakt door de teen- en oorknip is nog nooit middels een systematisch review onderzocht. We zullen daarom een systematisch review uitvoeren van het bewijs over ongerief ten gevolge van de oor- of teenknip, om dieronderzoekers, welzijn medewerkers, beleidsmakers en andere betrokkenen beter te informeren bij het maken van beslissingen over de keuze van de identificatiemethode voor knaagdieren.

Materiaal en Methoden
Review protocol en amendementen
De methodologie van het review is vooraf vastgelegd in een review protocol (zie bijlage). Naast de daarin beschreven methodologie hebben we 1) gezocht naar grijze literatuur in Google, OpenGrey.eu en WorldWideScience.org met behulp van alle mogelijke combinaties van de zoektermen 'muis', 'rat' of 'knaagdieren' met 'teen', 'oor', 'falanx' of 'clip' en 2) studies waarin een oorlabel werd aangebracht geïncludeerd, omdat ook dit een relevante identificatiemethode is. Vanwege de aanwezigheid van een oorlabel, kunnen deze resultaten niet worden gepooled met de studies zonder oorlabel.

Zoeken en studie selectie
We doorzochten de databases Pubmed, EMBASE en Web of Science met een zoekstrategie bestaande uit de volgende componenten: 'teen, staart of oor', 'ongerief' en 'dier' (zie bijlage voor volledige zoekstrategie). We controleerden de referentie-lijsten van alle geincludeerde studies en relevante reviews voor extra referenties die mogelijk relevant waren. Daarnaast hebben we grijze

literatuur doorzocht (zie protocol en amendement) en we hebben contact opgenomen met de Nederlandse vertegenwoordigers van de Federation of European Laboratory Animal Science Associations (FELASA), de vakgroep van proefdierdeskundigen en professoren in de proefdierkunde in Nederland met een verzoek om ons te informeren over al dan niet gepubliceerde gegevens over dit onderwerp.

Data-extractie
De gegevens werden geëxtraheerd door één reviewer (MB of FG) en gecontroleerd door een tweede reviewer (KW). We hebben geprobeerd om contact op te nemen met de auteurs van acht studies om aanvullende informatie over studie kenmerken en / of het resultaat gegevens te verstrekken. We kregen reacties van drie auteurs, die (gedeeltelijk) verduidelijking gaven m.b.t. studie kenmerken. Aanvullende data werden verstrekt door één van deze auteurs.

Risico op bias en kwaliteitsbeoordeling
Voor studies met een aparte controle groep beoordeelden twee onafhankelijke reviewers (KW en MB) het risico op bias en de studiekwaliteit, met behulp van SYRCLE's risk of bias tool. In geval van discrepanties werd consensus bereikt door discussie. Selectieve uitkomst rapportage (item # 9) werd niet beoordeeld, aangezien geen van de geïncludeerde studies refereerden aan een vooropgezet studieprotocol waarin de primaire en secundaire uitkomsten waren vastgelegd. Bij de beoordeling van selectie bias (item # 3), werden groepen binnen een studie bij aanvang vergelijkbaar beschouwd als het geslacht, de stam, en de leeftijd en / of het gewicht van de dieren niet significant verschilde tussen de groepen. Voor de teenknip studies waren een vergelijkbaar gewicht én leeftijd nodig, omdat de snelle ontwikkeling van pups grote verschillen tussen dieren van verschillende leeftijden kan veroorzaken en het gewicht van de pups invloed heeft op de nauwkeurigheid waarmee de teenknip kan worden uitgevoerd. Voor de oorknip studies was een vergelijkbaar gewicht óf leeftijd voldoende. Ook hebben we gekeken naar het rapporteren van enige vorm van randomisatie, blindering en een sample size berekening als aanvullende kwaliteitsindicatoren.
Omdat de risk of bias tool is ontwikkeld voor studies met aparte controle en experimentele groepen, konden vier studies niet worden gescoord vanwege incompatibele studie designs (dat wil zeggen cross-over design, of het gebruik van interne controles).

Her-analyse van de uitkomst data
Wanneer volledige uitkomst data konden worden geëxtraheerd of verkregen (d.w.z. gemiddelde, variantie en het aantal dieren per groep voor continue uitkomstmaten, of het aantal events en non-events voor dichotome uitkomstmaten) berekenden we de effect size als een standardised mean difference (SMD) of risico ratio (RR), voor respectievelijk continue en dichotome uitkomstmaten. Ons doel was een gecombineerd effect te berekenen bij drie of meer studies. Echter, geen enkele uitkomstmaat werd door meer dan twee studies gerapporteerd, en de resultaten en de studie kenmerken waren te heterogeen om verschillende uitkomstmaten te poolen. Daarom worden alleen de SMD en RR met bijbehorende 95% betrouwbaarheidsintervallen voor elke uitkomstmaat weergegeven zonder een gepoolde effectschatter. De SMDs en RRs werden berekend met een random effects model.

Resultaten
Studie selectie
We identificeerden in totaal 2040 referenties, waarvan er uiteindelijk twaalf voldeden aan de inclusiecriteria. Twee *conference abstracts* voldeden aan de inclusiecriteria, maar werden uitgesloten omdat de gepresenteerde gegevens vrijwel geheel overeenkwamen met volledige artikelen van dezelfde auteurs (we kregen geen respons op onze poging dit bij de auteurs te verifiëren). Daarbij werden de referentielijsten van 48 potentieel relevante referenties handmatig doorzocht en beoordeeld op relevantie. Geen van deze referenties voldeden aan de inclusiecriteria. Een conference abstract leek relevant te zijn, maar bevatte niet genoeg informatie om te beoordelen of

er een geschikte controlegroep werd gebruikt. We ontvingen helaas geen reactie toen we de auteurs benaderden voor aanvullende gegevens.

Studie karakteristieken

De karakteristieken van de studies zijn samengevat in tabel 1. Van de twaalf geïncludeerde studies onderzochten er vijf (42%) een oorknip zonder oorlabel, twee (17%) een oorknip met oorlabel en vijf (42%) een teenknip. Tien studies (83%) werden uitgevoerd in verschillende muisstammen, en twee in ratten (één oorknip en één teenknip). De meeste studies (58%) gebruikten zowel mannelijke als vrouwelijke muizen, en een aantal van deze studies presenteerde deze gegevens afzonderlijk voor beide seksen. In drie onderzoeken werd het geslacht van de dieren niet gemeld. In de oorknip (inclusief oorlabel) studies waren de dieren adolescent of volwassen op het moment van de interventie (3-12 weken voor muizen en 25 weken voor ratten). In de teenknip studies varieerde de leeftijd van postnatale dag (PND) 3 tot PND17 (mediaan PND7). De studies overlappen elkaar dus niet wat betreft de leeftijd van de dieren.

De meeste studies vergeleken uitkomstmaten in de geknipte groep met een controlegroep waarbij de dieren alleen gefixeerd werden. Drie studies gebruikten het ongeknipte, contralaterale oor of de ongeknipte teen als interne controle. Een studie beschreef de controle groep als "onbehandeld", maar of de dieren hierbij wel of niet werden gehanteerd of gefixeerd werd niet gespecificeerd. In twee studies vond een secundaire interventie in de controlegroep plaats naast het fixeren van de dieren (namelijk subcutane punctie en micro-tattoo van het pootje), zodat de controle groep beter overeenkomt met andere experimentele groepen in de studie. Deze extra interventies kunnen de mate van ongerief in de controlegroep hebben verhoogd, waardoor ze interfereren met de vergelijking tussen controle en oor- en teenknip. De plaats van het knippen is redelijk goed beschreven in alle studies, maar heterogeen: het pootje dat werd gekozen voor de teenknip verschilde tussen de studies. In alle oorknip studies werd de oorschelp geperforeerd, behalve in één studie waarin 2mm werd afgeknipt van de rand van het oor. Het aantal oren dat geknipt werd was 1-2. Het aantal tenen 1-3.

Uitkomstdata

De geïncludeerde studies beschrijven een grote verscheidenheid aan uitkomstmaten gerelateerd aan dierenwelzijn en ongerief. In tabel 1 staan deze uitkomstmaten genoemd en de richting van hun effect zoals beschreven door de auteurs. In veel studies werd een groot aantal uitkomstmaten bepaald, maar de precieze uitkomstdata werden vaak niet gerapporteerd of enkel beschrijvend weergegeven (ND in tabel 1).

Als een uitkomstmaat verschillende keren gemeten werd in hetzelfde dier, hebben we bij de her-analyse de meting met het maximale effect bepaald en weergegeven. De figuren 3-7 bevatten daarom de volgende data uit studies met herhaalde metingen: 1) Castelhano-Carlos2010: gewicht op PND21 (voor het spenen), gewicht in week 4 voor mannen en week 12 voor vrouwen (na het spenen); 2) Paluch2014: gewicht in week 9 voor mannen geknipt op PND7 en PND17, week 7 voor vrouwen geknipt op PND7 en week 10 voor vrouwen geknipt op PND17; 3) Castelhano-Carlos2010: rotarod test bij snelheid van 15rpm; 5) Vachon1998: lengte van de 3^{e} teen en breedte van de 4^{e} teen; 6) Kitagaki2007: oordikte na 26 weken en 7) Kasanen2011: hartslag op 16-24 uur en bloeddruk 4 tot 16 uur na oorknip.

Oorknip studies

In de oorknip studies werden vijftien verschillende uitkomstmaten (gemeten bij volwassenen dieren) beschreven, waarvan de meeste fysiologische parameters zijn voor ongerief zijn (bijvoorbeeld verhoogde hartslag en ontsteking). Twee gedragsparameters welke kunnen wijzen op ongerief of pijn werden beschreven (de *mouse grimace scale* en vocalisatie (piepen) tijdens behandeling). Muizen piepten vaker tijdens de oorknip dan tijdens enkel fixeren en hun respiratoir minuut volume steeg. Er werden geen verschillen in de hartslag, bloeddruk, lichaamstemperatuur en scores op de *mouse grimace scale* gezien. Het oormerken met metalen labels bleek het metaalgehalte van het oor

te verhogen wat ontsteking van het oorkraakbeen veroorzaakte, zoals aangetoond door een toename van de oordikte en een verhoogd cytokine niveau. Er werd geen effect op tumorvorming gezien. Bij ratten werd de bloeddruk en hartslag vergeleken tussen de oorknip en de microtattoo, met wisselende resultaten: de bloeddruk nam toe na de oorknip op verschillende tijdstippen, terwijl de hartslag hoger was in de microtattoo groep.
Bij de her-analyse van de data en het daarbij berekenen van de standardised mean difference (SMD; figuur 2) of het risico ratio (RR; figuur 3), vonden we geen extra effecten van de oorknip.

Teenknip studies
In de teenknip studies werden in totaal bijna vijftig verschillende uitkomstmaten beschreven (tabel 1). Uitkomstmaten gemeten in pups zijn onder te verdelen in parameters voor fysieke ontwikkeling (bijvoorbeeld lichaamsgewicht en ontwikkeling van vacht), neurologische ontwikkeling (bijvoorbeeld houdings- en grijpreflexen), tekenen van stress in pups of hun moeder (bijvoorbeeld vocalisatie tijdens behandeling en verstoting door de moeder) en fysiologische parameters die kunnen wijzen op ongerief (bijvoorbeeld een verhoogd corticosteroïd niveau en bloeding). Uitkomstmaten gemeten bij volwassen dieren betreffen voornamelijk neurologische testen en gedragstesten (bijvoorbeeld balanstest en open veld tests).
Het merendeel van de uitkomstmaten verschilt niet tussen teenknip en controles (tabel 1). Bij rattenpups geknipt op PND4 waren de prestaties in de wire suspension test op PND21 verminderd, wat een lagere grijpkracht indiceert. Een verminderde grijpkracht werd ook waargenomen in volwassen muizen die werden geknipt als pups op PND3, maar niet in muizen geknipt op PND7. Er werd geen teruggroei van tenen gezien en de dikte van het bot in de teen was toegenomen in de teenstompjes.
Bij her-analyse van de primaire data van de geïncludeerde studies (berekend als SMD of RR), vonden we vijf extra significante effecten van de teenknip, namelijk: toename vocalisatie, verminderde motorische activiteit en zwelling na het knippen op PND7 bij muizen, verminderd zwemvermogen als volwassen dier bij ratten geknipt op PND4 (zie figuur 5), alsmede een significante afname van het gewicht (na spenen) voor vrouwelijke pups geknipt op PND7 (figuur 4).

Kwaliteitsbeoordeling
Het risico op bias en de kwaliteit scores van de acht studies met aparte controlegroepen zijn weergegeven in tabel 2 (individuele scores) en figuur 6 (totaalscores). Hoewel randomisatie van toewijzing van dieren aan de experimentele groepen in zeven van deze studies (87,5%; figuur 6A) werd genoemd, werd de methode van randomisatie in geen enkele studie gespecificeerd. In drie van de acht studies (37,5%; figuur 6A) werd vermeld dat de uitvoerder van de experimenten (deels) geblindeerd was voor de behandeling, of dat hij/zij de uitkomstmetingen verrichtte alvorens te controleren tot welke experimentele groep het dier behoorde. In de overige studies werd blindering in geen enkele fase van het experiment genoemd. Géén van de twaalf geïncludeerde studies rapporteerde een onderbouwing van de steekproefgrootte of een powerberekening.
Vanwege de summiere rapportage van maatregelen om bias te verminderen, werden de meeste onderdelen van de risk of bias tool beoordeeld als een onduidelijk risico op bias (figuur 6B). Onvolledige rapportage van randomisering en/of de gebruikte methode leidt tot een onduidelijke risico op selectie, performance en detectie bias (items # 1, 4 en 6). De eigenschappen van de dieren aan het begin van de studie werden adequaat vermeld in twee studies, waarin we de kans op selectie bias als laag hebben geschat (item # 2). In de overige studies werden één of meer eigenschappen niet gemeld, wat leidt tot een onduidelijke risico op bias. Het risico op performance bias (item #5) was in alle studies hoog, omdat oor- en teenknippen in de praktijk onmogelijk te verbergen zijn bij zowel het uitvoeren van de ingreep als het hanteren van het dier daarna. Om deze reden hebben we ook het risico op detectie bias (item #7) hoog gescoord in alle studies, met uitzondering van die studies waarin de dieren niet per definitie werden gehanteerd voor bepaling van de uitkomstmaat. In deze laatste studies was echter onduidelijk of de uitkomstmaat daadwerkelijk geblindeerd was, daarom werden hier een onduidelijk risico op bias gescoord. Attrition bias (item #8) was hoog in één studie,

waar het aantal dieren beschreven in de materiaal en methoden niet overeenkwam met dat in sommige van de analyses. Twee studies rapporteerden de uitval van dieren op juiste wijze, waardoor een laag risico op attrition bias werd gescoord. In de resterende vijf studies was het risico op attrition bias onduidelijk. Het risico op andere soorten bias (item # 9) werd laag gescoord in alle studies.

Discussie

Vanwege het verwachte effect op het welzijn van dieren worden de teen- en oorknip over het algemeen beschouwd als controversiele technieken en is hun toepassing beperkt of zelfs afgeschaft in veel dierenlaboratoria. Een overvloed aan richtlijnen is beschikbaar over de teen- en oorknip, alsmede andere methoden voor identificatie en genotypering (e.g. 13-17). Echter, deze richtlijnen zijn tot nu toe niet gebaseerd op een systematisch review van al het beschikbare wetenschappelijke bewijs. Wij voerden het eerste systematische review uit van het bewijs naar het effect van de teen- en oorknip op het welzijn van knaagdieren.

Beschikbaar bewijs en kwaliteit

Studies die onderzoek doen naar de effecten van de teen- en oorknip op het welzijn van knaagdieren zijn schaars en erg heterogeen. Deze heterogeniteit wordt voornamelijk veroorzaakt door verschillen in de populatie (geslacht, stam) en de interventie (leeftijd op moment van knippen, aantal plaatsen geknipt), evenals het grote aantal verschillende uitkomstmaten dat gemeten is. De meeste uitkomstmaten toonden geen effect van teen- of oorknip op ongerief aan. Aan de andere kant zijn er ook uitkomstmaten die wel bewijs voor ongerief aantonen, voor zowel de oorknip (verhoogd ademvolume, vocalisatie en bloeddruk) als voor de teenknip (meer vocalisatie, minder activiteit in pups en minder grijpkracht en zwemvermogen in volwassenen). Echter, meerdere beperkingen van de primaire studies verminderen de betrouwbaarheid van zowel het bewijs vóór als tegen een effect op ongerief en bemoeilijken de interpretatie ervan.

Allereerst is adequate rapportage van de experimentele methode cruciaal om het risico op bias en de kwaliteit van de studies te bepalen. Onze kwaliteitsbeoordeling laat zien dat de rapportage van de dierstudies ontoereikend is. Daardoor werd het risico op de meeste vormen van bias als onduidelijk gescoord. Dit is een punt van zorg, omdat is aangetoond dat het ontbreken van (rapportage van) methoden om bias te verminderen de studieresultaten ernstig kan beïnvloeden. In alle geïncludeerde studies was sprake van een hoog risico op performance en detection bias, waarmee rekening gehouden moet worden bij het interpreteren van de resultaten.

Het specificeren van een primaire uitkomstmaat voorkomt het aanpassen van de uitkomstmaat op basis van de studieresultaten en daarmee het risico op bias. Echter, geen van de studies definieerde welke uitkomstmaat de primaire uitkomstmaat was. Ook werd er in geen van de studies een power berekening gerapporteerd, terwijl een onderbouwing van het aantal dieren per groep door veel ethische commissies verplicht wordt gesteld. In de powerberekening moeten de primaire uitkomstmaat, het verwachtte gemiddelde en de variatie, en het effect dat de auteurs willen detecteren gespecificieerd zijn. Dit voorkomt het onnodig en daarmee onethisch gebruik van te veel of te weinig dieren. Ook kan de lezer de powerberekening gebruiken om te beoordelen of de uitval van dieren op de juiste wijze is gerapporteerd en wat het risico op attrition bias is. Op dit moment is het onmogelijk om te bepalen of de steekproefgrootte in een van de studies toereikend was om een verschil tussen te experimentele groepen aan te tonen.

In één studie was het onduidelijk welke interventie in de controle groep werd toegepast, wat de interpretatie van de resultaten van deze studie bemoeilijkt. In twee andere studies werd een tweede interventie in de controle groep toegepast, die mogelijk meer ongerief bij de dieren gaf en daarmee het effect van de teen- of oorknip kan hebben gemaskeerd. Eén studie gebruikte een cross-over design, waar bij *carry-over* effecten kunnen optreden die interfereren met de onderzochte interventie. Daarnaast tonen we aan dat de data van verschillende uitkomstmaten onvolledig zijn gerapporteerd. Al deze tekortkomingen worden waarschijnlijk (mede) veroorzaakt worden door het feit dat sommige studies niet specifiek ontworpen waren om het effect van de teen- of oorknip te

vergelijken met een controlegroep. Echter, dit illustreert nogmaals aan dat studies specifiek ontworpen om het effect van teenknip of oorknip op dierenwelzijn erg schaars zijn.
Als gevolg van bovengenoemde kanttekeningen zijn de resultaten van de verschillende studies niet direct vergelijkbaar met elkaar, en kunnen de resultaten niet geëxtrapoleerd worden naar knaagdieren in het algemeen. Totdat er meer betrouwbare gegevens beschikbaar zijn kan een effect van teenknip of oorknip op het dierenwelzijn en de onderzoeksresultaten niet worden uitgesloten of bevestigd.

Mogelijkheden voor verfijning
Het bestaande bewijs is te beperkt en te heterogeen om betrouwbaar de invloed van bijvoorbeeld diersoort, leeftijd, geslacht, ras, of de methode van knippen op de ernst van het ongerief te beoordelen. Drie studies deden afzonderlijke analyses voor mannen en vrouwen, maar de effecten verschilden niet tussen de verschillende sexen. Schaeffer et al. vonden dat pups die een teenknip ondergingen op PND3 een lagere grijpkracht hadden dan pups geknipt op PND7. Dit werd toegeschreven aan het feit dat de tenen op PND3 nog deels vergroeid zijn en te klein zijn om nauwkeurig te knippen, waardoor er een te groot deel van de teen verwijderd wordt. Op basis hiervan zou het knippen van de teen voor PND3 niet aan te raden zijn, maar replicatie van dit onderzoek is nodig om dit te bevestigen. Studies waarin de teenknip uitgevoerd werd op PND4 en PND5 rapporteerden dat de procedure snel en eenvoudig toe te passen was, terwijl onze heranalyse van de data van Paluch et al. suggereert dat er ongerief optreed in zeven dagen oude pups (toename vocalisatie, verminderde motorische activiteit), maar niet in zeventien dagen oude pups. Hieruit concluderen we dat het effect van leeftijd onduidelijk is en dat er verder onderzoek op dit gebied nodig is.
Eén studie onderzocht het toepassen van pijnstillende vapocoolant spray tijdens de teenknip, maar rapporteerde dat deze spray tenen aan elkaar plakte waardoor het risico op onjuist knippen toenam en er een toename was van ongerief en stress door het langduriger hanteren van de dieren. Ook verstoorde de spray de bloedstolling na het knippen. Op basis hiervan is het toepassen van deze pijnstillende spray niet te adviseren, maar verder onderzoek naar mogelijke andere pijnstillers of locale anesthetica kunnen de moeite waard zijn.

Implicaties voor de praktijk
De voor- en nadelen van de teen- en oorknip en diverse andere identificatiemethoden zijn uitvoerig beschreven door verschillende specialistische onderzoeks- en werkgroepen zoals de FELASA, het Noorse Concensus Platform voor vervanging, vermindering en verfijning van dierproeven (Norecopa) en de gezamenlijke BVAAWF/FRAME/RSPCA/UFAW werkgroep. In het rapport van Norecopa uit 2008 wordt gemeld dat ze geen studies hebben gevonden met fysiologisch of histologisch bewijs waarop beoordeeld zou kunnen worden of pups pijn kunnen ervaren op de leeftijd waarop de teenknip wordt toegepast. Dergelijke studies vonden wij ook niet middels onze systematische zoekstrategie.
Op basis van de beschikbare richtlijnen is er consensus dat de teenknip niet uitgevoerd moet worden na PND7, omdat pups naarmate ze ouder worden steeds actiever worden waardoor het risico op het onjuist knippen en de noodzaak tot fixeren van het dier toeneemt. Dit staat los van de observatie dat botvorming in de teenkootjes voltooid is rond PND18, waarvan verondersteld wordt dat het knippen daarna pijnlijker zal zijn. We vonden echter geen data die deze theorie ondersteunt. Daarentegen wordt het toepassen van de oorknip juist afgeraden vóór PND14, omdat het oor voor die tijd nog te klein is. De oor- en teenknip kunnen dus niet op dezelfde leeftijd worden toegepast en de leeftijd waarop de identificatie nodig is, is dus een essentiële factor bij de keuze voor oor- of teenknip. Dit geldt ook voor de verschillende onderzochte alternatieven, zoals tattoo of microchip voor identificatie en haar of slijmvlies biopten voor genotypering. De meeste van deze technieken kunnen niet worden gebruikt bij pasgeboren of zeer jonge dieren en kunnen daarom de teenknip niet vervangen. Bovendien lijken sommige van deze technieken meer ongerief te veroorzaken dan de oor-

of teenknip. Een andere factor die de identificatiemethode bepaalt is of (en hoeveel) DNA nodig is voor (kwantitatieve of kwalitatieve) genotypering, en of de identificatie permanent of tijdelijk moet zijn.

Toekomstig onderzoek

Dit review toont een aantal belangrijke tekortkomingen van het beschikbare wetenschappelijk bewijs aan die de interpretatie ervan belemmeren: 1) het geringe aantal studies dat specifiek de invloed van de oor- of teenknip op ongerief heeft onderzocht, 2) het ontbreken van standaardisatie van de uitkomstmaten, 3) onvoldoende (gedetailleerde) rapportage van de onderzoeksmethodologie, in het bijzonder de powerberekening en methoden om bias te verminderen en 4) onvolledige rapportage van uitkomstdata. Daarom is er meer onderzoek nodig om betrouwbaar bewijs van het effect van oor- of teenknip op dierenwelzijn te verkrijgen. Alle hierboven genoemde tekortkomingen moeten in toekomstige studies worden gecorrigeerd.

Ten eerste zullen de toekomstige studies specifiek gericht moeten zijn op vaststellen van het effect van de oor- of teenknip op ongerief bij knaagdieren. Daarvoor moeten ze een passende controlegroep hebben, bij voorkeur één groep waarin dieren geen enkele behandeling ondergaan (ten einde een baselinemeting te verkrijgen) en één waarin dieren een sham behandeling ondergaan waarbij ze gehanteerd en gefixeerd worden. Er dienen geen co-interventies toegepast te worden. De dieren dienen onder dezelfde omstandigheden behandeld en gehuisvest te worden. Verder is het onduidelijk of karakteristieken zoals diersoort, stam, geslacht en leeftijd van invloed zijn op de uitkomstmaten. Toekomstig onderzoek zal specifiek opgezet moeten zijn om dit betrouwbaar te kunnen onderzoeken.

Ten tweede is het noodzakelijk dat onderzoekers al bij het indienen van een voorstel voor nieuwe dierexperimenten een argumentatie voor de gekozen uitkomstmaten geven, inclusief gegevens over reproduceerbaarheid en het optimale tijdstip van meten. Idealiter zou de relevantie en de reproduceerbaarheid van de uitkomstmaten betreffende het beoordelen van ongerief bij pups en volwassen knaagdieren gevalideerd en bediscussieerd worden onder de deskundigen zodat methoden gestandaardiseerd kunnen worden. Multi-center studies kunnen mogelijkheden bieden om de power en standaardisering van toekomstige experimenten te vergroten.

Tot slot laten we in dit review de noodzaak zien van het verbeteren van (de rapportage van) de methodologische kwaliteit van de dierstudies. De ARRIVE richtlijn en de Gold Standard Publication Checklist (GSPC) werden in 2010 gepubliceerd, maar de kwaliteit van de geïncludeerde studies was laag, ongeacht het jaar van publicatie. We wijzen erop dat de ARRIVE richtlijn en de GSPC niet specificeren hoe gedetailleerd de gebruikte methoden om bias te verminderen gerapporteerd moeten worden. De risk of bias tool van SYRCLE geeft aanwijzingen voor het rapporteren van deze methoden in verschillende stadia van een experiment. Dit is voor onderzoek naar de oor- of teenknip van groot belang, omdat deze interventies moeilijk te blinderen zijn, waardoor het risico op bias groot is. Het rapporteren van een powerberekening zou verplicht moeten zijn voor toekomstige studies. Tot slot is complete rapportage van de data van alle uitkomstmaten, in het artikel, bijlagen, of in *data repositories*, essentieel om in de toekomst betrouwbare conclusies te kunnen trekken in het belang van wetenschap en dierenwelzijn.

3b

Systematic review of discomfort due to toe or ear clipping in laboratory rodents.

Kimberley E Wever[1], Florentine J Geessink[1], Michelle Brouwer[1], Alice Tillema[2] and Merel Ritskes-Hoitinga[1]

[1]SYstematic Review Centre for Laboratory animal Experimentation (SYRCLE) and [2]Medical Library, Radboud university medical center, Nijmegen, The Netherlands

Corresponding author:
Dr. K.E. Wever
SYstematic Review Centre for Laboratory animal Experimentation (SYRCLE)
Radboud university medical center, Nijmegen, The Netherlands.
Street address: Geert Grooteplein-Noord 29, route 231, 6525 GA Nijmegen, The Netherlands
Postal address: PO Box 9101, 6500 HB Nijmegen, The Netherlands
E-mail: kim.wever@radboudumc.nl
Phone: +31 (0)24 36 68364
Fax: +31 (0)24 36 11068

1 Abbreviations

2 PND – Post natal day

3 RR – Risk ratio

4 SMD – standardized mean difference

Abstract

Reliable identification of individual laboratory animals is an elementary requirement in many animal experiments. Identification methods should be effective, practical, and cause minimal distress to the animal, so that they do not cause unwanted variability in the experiment. Toe clipping and ear clipping are frequently used for individual identification and genotyping of mice and rats. These procedures are expected to have a large impact on animal wellbeing. However, no systematic summary of the evidence on this topic currently exists. We conducted a systematic review of the evidence for discomfort due to toe clipping and ear clipping, in order to better inform animal researchers and other stakeholders involved in the choice of identification methods for rodents.

Through a systematic search in three databases, we identified seven studies on the effect of ear clipping and five studies on the effect of toe clipping on animal welfare. Two of these studies were performed in rats, the others in mice. The study characteristics were highly heterogeneous in terms of age, strain and sex of the animals, the timing of the intervention and the number of toe or ear clips. Our risk of bias assessment showed that there was insufficient reporting of measures to reduce bias, leading to an unclear or high risk of bias in all studies. None of the studies reported a power calculation to justify the chosen sample size. In addition, a multitude of outcome measures related to animal welfare was reported (nearly fifty and fifteen different outcomes in toe clip and ear clip studies, respectively). Out of all of these outcomes, we found evidence for discomfort due to ear clipping in the form of increased respiratory volume, vocalization and blood pressure. For toe clipping, increased vocalization and decreased motor activity in pups was found, as well as long-term effects in the form of reduced grip strength and swimming ability in adults.

In conclusion, there is too little evidence to reliably assess discomfort due to toe or ear clipping and the quality of the available evidence is uncertain. Adequately powered, high-quality studies reporting reliable, relevant outcome measures are needed to accurately assess the impact of these identification techniques. Until more reliable evidence is available, an effect of toe clipping or ear clipping on animal welfare and study results cannot be excluded or confirmed.

1. Introduction

Rodents, especially mice and rats, are the most frequently used laboratory mammals in biomedical research. They are often very similar in appearance, and are usually housed in groups for practical and welfare reasons. Individual identification of the animals is often necessary during breeding, daily care or experimental procedures, and several identification methods are in regular use. Selection of the best method of individual identification depends on several factors, including species, age, skin pigmentation, study duration, and the technical expertise available. The ideal identification method should be effective and practical, but should also be minimally invasive in terms of pain and/or distress to the animal, since this can reduce animal welfare and distort the experimental results. It is therefore important to assess the effect of identification methods on animal wellbeing.

Toe clipping is an individual identification method mostly used in mice, which can be applied in newborn and very young animals. The toe may be clipped at the distal end of the second phalanx to remove the entire nail bed, or a larger segment of the toe may be removed. The removed tissue can be used for genotyping. Ear clipping or punching (notching) is used to identify individual adult rodents (mostly mice and rats). Using a special puncher, holes or notches are made in the ear according to a chart/system. The punched or clipped tissue can be used for genotyping.

The ethical justification to perform these methods is a matter of debate, since both methods are likely to cause pain and/or distress. Both methods require restraint of the animals and may permanently affect the wellbeing of the animal. For instance, toe clipping might impair the mouse's ability to grip, groom and feed, as well as altering the animal's gait. Ear clipping may interfere with thermoregulation and clipped ears may be more susceptible to tearing and infection. Although many guidelines on the subject are available, the evidence for the discomfort caused by toe or ear clipping has not been systematically reviewed. We have therefore conducted a systematic review of the evidence on discomfort due to ear and toe clipping, in order to better inform animal researchers,

welfare officers, policy makers and other stakeholders when making decisions on the choice of identification method for rodents.

2. Materials and methods

2.1. Review protocol and amendments

The review methodology was pre-specified in a review protocol (supplemental material). We made minor amendments to the review protocol: 1) We searched for grey literature in Google, OpenGrey.eu and WorldWideScience.org, using all possible combinations of the search terms "mouse", "rat" or "rodent" with "toe", "ear", "phalanx" or "clip", 2) Studies applying ear tags were included in the review, since the ear is punched in this procedure and ear tagging is also a relevant identification method. However, because of the presence of a tag, results of these studies cannot be pooled with ear clip studies in which no tag is applied.

2.2. Search and study selection

See supplemental material for the full search strategy. In brief, we performed a comprehensive search in Pubmed, EMBASE and Web of Science, using the search components "toe, tail or ear", "discomfort" and "animal". We checked the reference lists of all included studies and relevant reviews for additional references of interest. In addition, we performed a grey literature search (see 2.1) and we contacted the Dutch representatives of the Federation of European Laboratory Animal Science Associations (FELASA), as well as animal welfare officers and professors in laboratory animal science in The Netherlands with a request to inform us about any published or unpublished data on this topic.

2.3. Data extraction

Data were extracted by one reviewer (MB or FG) and checked by a second reviewer (KW). We attempted to contact the authors of eight studies to provide additional information on study

characteristics and/or outcome data. We received responses from three authors, who were able to (partially) clarify study characteristics. Additional outcome data were provided by one author.

2.4. Risk of bias and quality assessment

For studies using a separate control group, two reviewers (KW and MB) independently assessed the risk of bias and study quality using SYRCLE's risk of bias tool [1]. In case of discrepancies, consensus was reached by discussion. Selective outcome reporting (item #9) was not assessed, since none of the studies reported the use of a study protocol predefining primary and secondary outcomes. When assessing selection bias (item #3), groups within a study were considered similar at baseline if the sex, strain, and age and/or weight of the animals did not significantly differ between groups. For toe clip studies, similar weight and age were required, because the fast development of pups can cause large difference between animals of different ages, and the weight of the pups influences the accuracy with which toe clipping can be performed. For the ear clip studies, similar weight or age was considered sufficient.

We also assessed reporting of any randomization, reporting of any blinding, and reporting of a sample size calculation as additional study quality indicators.

Because the risk of bias tool was developed for studies using separate control and treated groups, four studies could not be scored due to incompatible study designs (*i.e.* cross-over design or use of internal controls).

2.5. Re-analysis of outcome data

Whenever complete outcome data could be extracted or obtained (*i.e.* mean, variance and number of animals per group for continuous outcomes, or the number of events and non-events for dichotomous outcomes) we re-analysed the data by calculating the effects size as a standardized mean difference (SMD) or risk ratio (RR), for continuous and dichotomous outcomes, respectively. We aimed to obtain pooled effect estimates of outcome measures reported by three or more

studies. However, no single outcome was reported more than twice, and outcome and study characteristics were too heterogeneous to pool various outcomes. We therefore only report the SMD and RR and corresponding 95% confidence intervals for the individual outcomes per study, without pooling data. Effect estimates were calculated using a random effects model.

3. Results

3.1. Study selection

A flow chart of the study selection process is depicted in Figure 1. We identified a total of 2040 unique references, twelve of which met the inclusion criteria. Two conference abstracts met the inclusion criteria, but were excluded because the data presented appeared to match those in included full research articles by the same authors (an attempt to contact the authors to verify this received no response). An additional 48 potentially relevant references were identified by hand-searching references lists of included studies and relevant reviews, and grey literature searching. None of these references met the inclusion criteria. One conference abstract appeared to be relevant, but did not contain enough information to assess whether an appropriate control group was used. The authors were contacted for additional data, but these were not supplied.

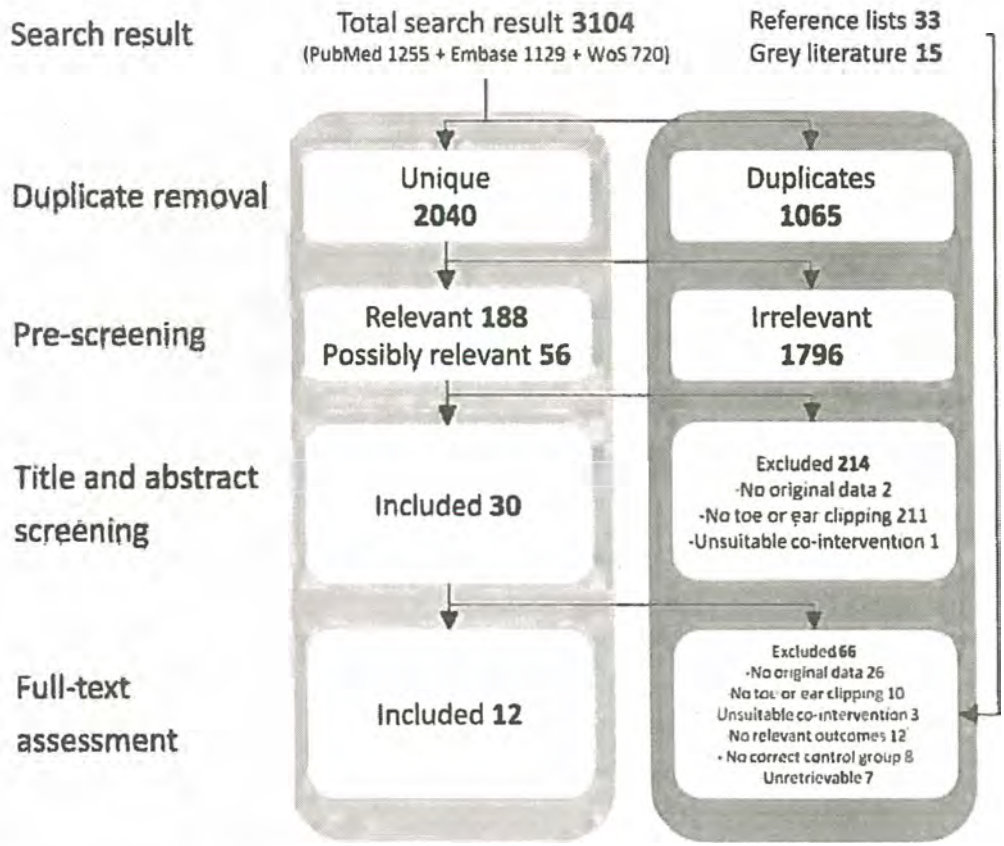

Fig 1 | Flow chart of the study selection process.

3.2. Study characteristics

The characteristics of the included studies are summarized in Table 1. Out of the twelve studies included, five (42%) were on ear clipping without tagging, two (17%) were on ear clipping with tagging, and five (42%) were on toe clipping. Ten (83%) studies were performed in varying strains of mice, and two studies were performed in rats (one on ear and one on toe clipping). The majority of studies (58%) used both male and female mice, and a number of these studies presented data separately for both sexes. In three studies, the sex of the animals was not reported. For ear clip (including ear tag) studies, the animals were adolescent or adult at the time of intervention (3-12 weeks for mice and 25 weeks for rats). In toe clip studies, the age at intervention ranged from postnatal day (PND) 3 to PND17 (median PND7). Thus, the age at the time of clipping did not overlap between ear and toe clip studies.

Table 1 | Characteristics of the included studies

Author + year	Species	Strain	Sex	Age at intervention	Intervention of interest	Site of clipping	Freq	Control intervention	Outcome [direction of effect*]
Cinelli2007[2]	Mouse	B6.Cg-Tg (ACTBBgeo / GFP)-21Lbe/J	m/f	12 wk	Ear clip	Pinna (2mm punch)	1	Restraint	In adults: Heart rate [=] Body temperature [=] Motor activity [=]
Kasanen2011[3]	Rat	SD / Wistar	NR	25 wk	Ear clip	Pinna, right	2	Foot micro-tattoo*	In adults: Heart rate 16-24h post-treatment [↓] Blood pressure 1-24h post-treatment [↑]
Miller2015[4]	Mouse	C57BL/6	m	8 wk	Ear clip	Pinna	1	Restraint	In adults: Mouse grimace scale directly post-treatment [=]
Rasid2012[5]	Mouse	Balb/c × TCR-HA+/–	m/f	8-10 wk	Ear clip	Pinna (2mm band)	1	Restraint	In adults: Respiratory minute volume [↑]
Williams2008[6]	Mouse	B6;129S6-Stat5b	m/f	PND 21-28	Ear clip	Pinna	1	Restraint	In adults: Vocalization during treatment [↑]
Baron2005[7]	Mouse	FVB/N / FVB/NCr	NR	3-4 wk	Ear tag	Pinna, right base	1	Contralateral ear	In adults: Tumor incidence [=] Gross histopathology [?] Grading of squamous cell carcinomas [?]
Kitagaki2007[8]	Mouse	C57BL/6	m	5 wk	Ear tag	Pinna, right base	1	Contralateral ear	In adults: Ear lesions [?] Thickness of ear auricles [↑] Ear metal content [↑] Cytokine levels [↑] Methallothionein expression (ND)[↑] Histopathological changes (ND)[?]

Table 1|Characteristics of the included studies

Author + year	Species	Strain	Sex	Age at interventio n	Intervention of interest	Site of clipping	Freq	Control intervention	Outcome [direction of effect*]
Castelhano-Carlos2010[9]	Mouse	C57BL/6J	m/f	PND 5	Toe clip	1/3 of a toe	1	Restraint and s.c. puncture	In pups: Nest disruption (ND)[=] Rejection by mother (ND)[=] Cannibalism (ND)[=] Urination upon treatment (ND)[=] Vocalization upon treatment (ND)[=] Urination and vocalization upon treatment [=] Skin appearance (ND)[=] Milk spots (ND)[=] Physical activity PND5 (ND)[=] Body weight [=] Anogenital distance (ND)[=] Development of fur (ND)[=] Negative geotaxis (ND)[=] Development of ears, eyes and teeth [=] Postural reflex, grasping reflex, surface righting reflex, wire suspension test and air-righting reflex (ND)[=] Mature walking (ND)[?] In adults: Body weight [=] Home-cage climbing behavior [=] Elevated plus-maze (ND)[=] Rotarod treadmill constant velocity [=] Rotarod treadmill accelerating velocity (ND)[=] Simplified SHIRPA protocol (ND)[=] Adrenal gland weight (ND)[=] Thymus weight (ND)[=]

Table 1|Characteristics of the included studies

Author + year	Species	Strain	Sex	Age at interventio n	Intervention of interest	Site of clipping	Freq	Control intervention	Outcome [direction of effect*]
Iwaki1989[10]	Rat	SD-JCL	m/f	PND 4	Toe clip	1st joint of a forelimb toe	1	Untreated**	In pups: Survival until weaning [=] Erection of pinnae PND7 [=] Righting reflex PND7 [=] Opening of eyelids PND17 [=] Negative geotaxis PND14 [=] Wire suspension PND21 [↓] Swimming ability PND21 [=] Rotarod treadmill PND28 [=] Sexual maturation [=] Body weight (ND)[=] In adults: Pregnancy success rate [=] Number of viable fetuses [=] Offspring fetal weight [=]

Table 1 | Characteristics of the included studies

Author + year	Species	Strain	Sex	Age at intervention	Intervention of interest	Site of clipping	Freq	Control intervention	Outcome [direction of effect*]
Paluch2014[11]	Mouse	C57BL/6J	m/f	PND7 / 17	Toe clip	Distal end of 1st phalanx	2 (1 fore, 1 hind)	Restraint	In pups: Vocalization upon treatment [? clipped at PND7; = clipped at PND17] Urination upon treatment [? clipped at PND7; = clipped at PND17] Struggle upon treatment [? clipped at PND7; = clipped at PND17] Dragging limb after treatment [? clipped at PND7; = clipped at PND17] Reduced activity directly after treatment [? clipped at PND7; = clipped at PND17] Suckling behavior (ND)[=] Maternal rejection (ND)[=] Paw swelling (ND)[?] Paw erythema (ND)[?] Body weight until 10 wk [=] Neurologic reflex development (negative geotaxis, surface righting reflex, grasping reflex, postural reflex, object grasping, wire suspension test, mature walking, air-righting reflex) (ND)[=] In adults: Open-field test (ND)[=] Elevated plus-maze test (ND)[=] Simplified SHIRPA protocol (ND)[=] Balance beam test (ND)[=] Rotarod treadmill (ND)[=]

Table 1|Characteristics of the included studies

Author + year	Species	Strain	Sex	Age at intervention	Intervention of interest	Site of clipping	Freq	Control intervention	Outcome [direction of effect*]
Schaefer2010[12]	Mouse	B6D2F1	m/f	PND 3 / 7	Toe clip	2nd phalanx	3 (2 fore, 1 hind)	Restraint	In pups: Paw withdrawal [?] Bleeding [=] Grooming by mother [=#] Cannibalism by mother [=#] Automutilation [=#] Inflammation [=#] Milk spots [=#] Righting [=#] Development of fur, teeth, ears, eyes and walking [=#] Body weight [=] Corticosterone levels [=] In adults: Grip strength [↓clipped at PND3; = clipped at PND7] Hot plate [= clipped at PND3; = clipped at PND7] Bone regeneration (ND)[=] Bone hypertrophy (ND)[=]
Vachon1998[13]	Mouse	C57BL, C3H, B6C3F1	NR	2 wk	Toe clip	Distal end of 1st phalanx	1	Contralateral toe(s)	In adults: Phalangeal bone length [↓] Phalangeal bone width [↑]

NR not reported; m male; f female; wk weeks; PND post-natal day; (ND) descriptive, data not shown, or comparison control *versus* clipped not analyzed
*As described by the authors, indicated as follows: ↑ clipping increases outcome; ↓ clipping decreases outcome; = clipping does not affect outcome; ? the outcome is assessed, but the effect of clipping *versus* control is not described; #Additional data provided by authors; **not reported whether the control group underwent restraint, handling or no intervention at all

Most studies compared outcomes in the clipped group(s) to a control group undergoing restraint only. Three studies used the unclipped contralateral ear[7,8] or toe[13] as an internal control. One study[10] described the control group as being "untreated", but whether this included handling or restraint of the animals was not specified. Two studies performed a secondary intervention in the control group in addition to restraint (namely subcutaneous puncture[9] and microtattoo of the foot[3]), in order to better match the control group with other experimental groups in the study. Importantly, these interventions may have increased the level of distress in the control group and therefore interfere with the comparison with the toe or ear clipped group. The site of clipping was reasonably well described, but heterogeneous: the paw chosen for toe clipping differed between studies. All ear clip studies performed ear punching, except for one in which a 2mm band was clipped off the rim of the ear[5]. The number of ear clips applied was 1-2. The number of clipped toes was 1-3.

3.3. Outcome data

The primary studies report a wide variety of outcome measures related to animal welfare and distress. Table 1 lists the outcomes and their direction of effect, as reported by the authors in the primary studies. In many studies, a large number of outcome measures was reported to be assessed, but the outcome data were often not shown, or reported descriptively (ND in table 1).

When an outcome was repeatedly measured in the same animals, we re-analyzed data from the measurement of maximal effect. Thus, figures 2-6 include the following data from studies with repeated measurements: 1) Castelhano-Carlos2010.: pre-weaning body weight on PND21, post-weaning body weight in week 4 for males and week 12 for females; 2) Paluch2014: body weight data in week 9 for males clipped at PND7 and PND17, week 7 for females clipped at PND7 and week 10 for females clipped at PND17; 3) Castelhano-Carlos2010: rotarod treadmill data 15rpm velocity; 5) Vachon1998: phalangeal length data of the 3rd digit and phalangeal width data of the 4th digit; 6)

Kitagaki2007: ear thickness at 26 weeks and 7) Kasanen2011: heart rate 16-24h and blood pressure 4-16h after ear clipping.

3.3.1. Ear clip studies

Overall, ear clip studies reported fifteen different outcomes (all measured in adults), the majority of which are physiological parameters related to discomfort (*e.g.* elevated heart rate and inflammation). Two behavioral parameters indicating discomfort or pain were reported (mouse grimace scale and vocalization during treatment).

Mice were reported to vocalize more frequently during ear clipping than during restraint only[6], and their respiratory minute volume was increased[5]. No differences in heart rate, blood pressure, body temperature and scores on the mouse grimace scale were reported[2,4]. Tagging with metal ear tags was found to increase the metal content of the ear and cause auricular chondritis[8], as indicated by an increase in ear thickness and elevated cytokine levels. No effect on tumor formation was observed[7]. In rats, blood pressure and heart rate were compared between ear clipping and foot microtattoo, with varying results: blood pressure was increased at various time points after ear clipping, while heart rate was higher in the micro-tattooed animals[3].

When re-analyzing the primary data from included studies as standardized mean difference (SMD; figure 2) or risk ratio (RR; figure 3), we found no additional effects of ear clipping.

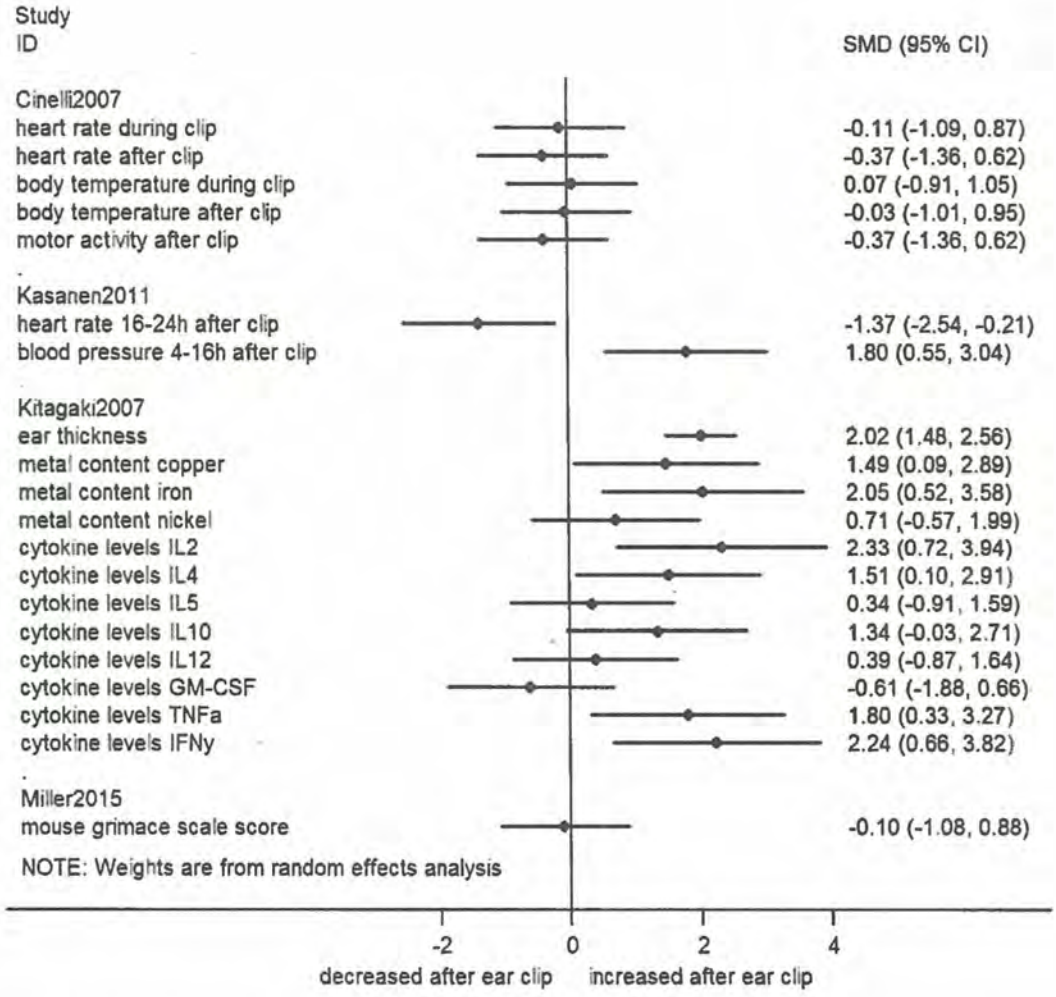

Fig 2|Forest plot of continuous outcome data from ear clip studies. Effect sizes calculated as standardized mean difference (SMD) and corresponding 95% confidence interval (CI), using a random effects model. H = hours; IL = interleukin; GM-CSF 5 = granulocyte macrophage colony-stimulating factor; TNFa = tumor-necrosis factor-α; IFNy = interferon-γ.

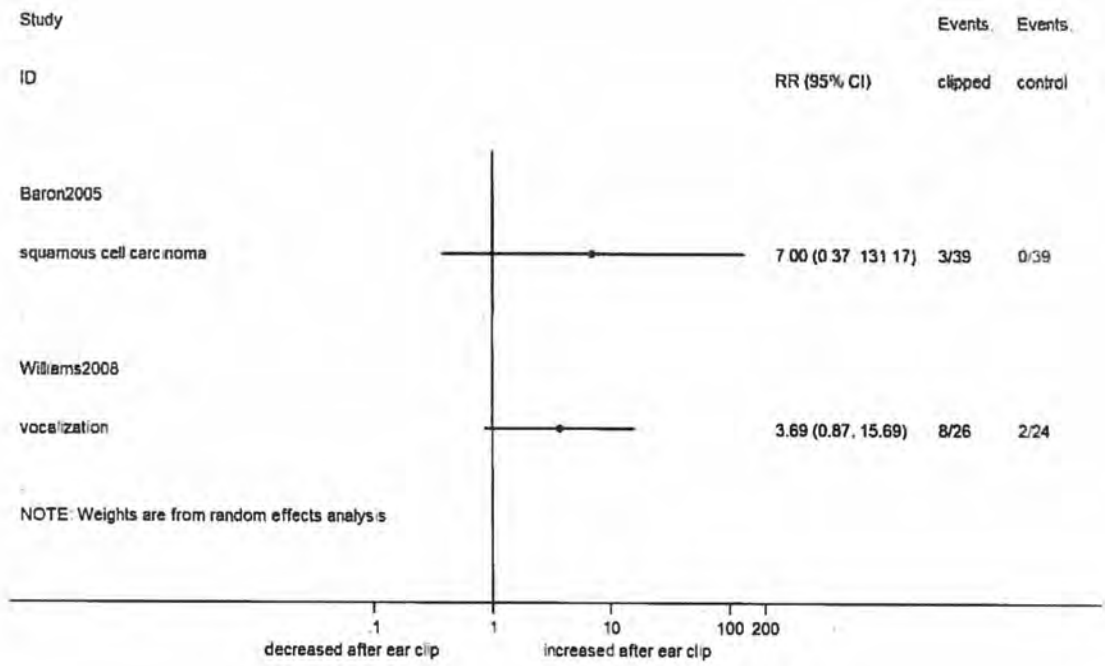

Fig 3|Forest plot of dichotomous outcome data from ear clip studies. Effect sizes calculated as risk ratio (RR) and corresponding 95% confidence interval (CI), using a random effects model. Right-hand side columns indicate events from total in treatment (clipped) and control groups.

3.3.2. Toe clip studies

Overall, toe clip studies reported on nearly 50 different outcome measures (table 1). Outcomes measured in pups can be divided into parameters related to physical development (*e.g.* body weight, development of fur and sexual maturation), neurological development (*e.g.* righting and grasping reflexes), signs of distress in pups or their mother (*e.g.* vocalization during treatment and maternal rejection) and physiological parameters indicating discomfort (*e.g.* elevated corticosteroid levels and bleeding). Outcomes measured in adult animals mainly include neurological and neurobehavioral tests (*e.g.* balance beam and open-field tests).

The majority of outcomes was reported to be unchanged between toe clipped and control animals (table 1). In rat pups clipped on PND4, performance in the wire suspension test on PND21 was found to be decreased, indicating lower grip strength[10]. Decreased grip strength was also observed in

adult mice that were clipped as pups on PND3, but not in mice clipped on PND7[12]. No regrowth of toes was reported and the thickness of the phalangeal bone was increased in toe stumps[13].

When re-analyzing the primary data from included studies as SMD or RR, we found five additional significant effects of toe clipping, namely: increased vocalization, reduced motor activity and toe swelling after clipping on PND7 in mice, impaired adult swimming ability in rats clipped on PND4 (all figure 5), as well as a borderline significant decrease in post-weaning body weight for female pups clipped on PND7 (figure 4).

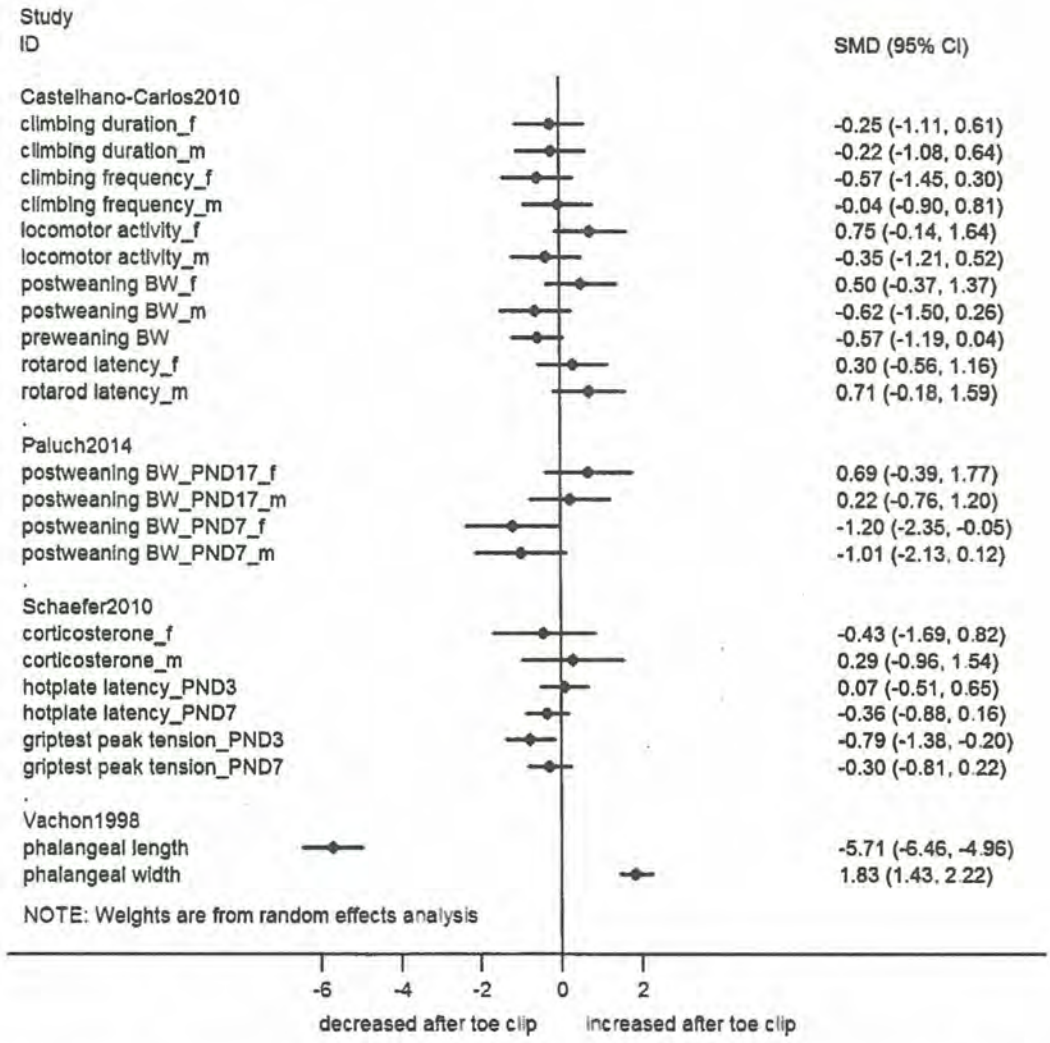

Fig 4|Forest plot of continuous outcome data from toe clip studies. Effect sizes calculated as standardized mean difference (SMD) and corresponding 95% confidence interval (CI), using a random effects model. f = female; m = male; BW = body weight; PND = postnatal day; h = hour.

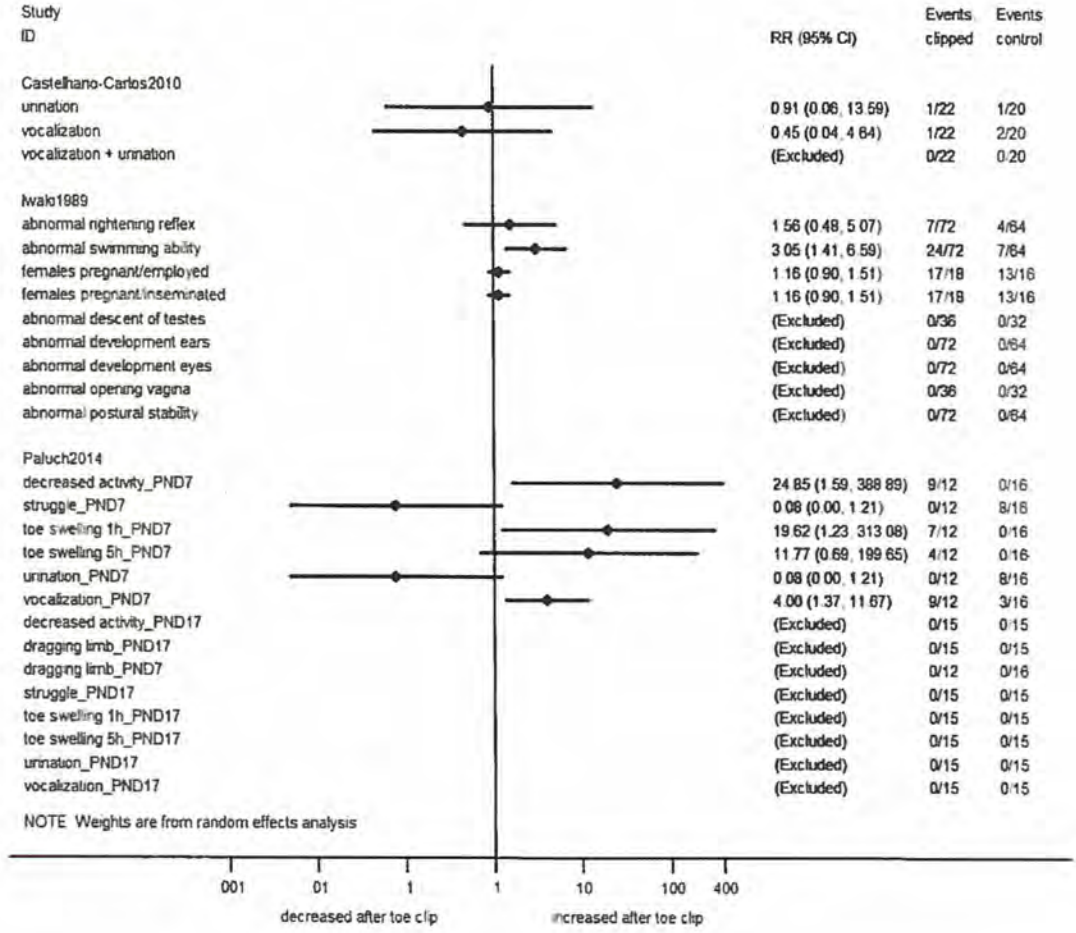

Fig 5|Forest plot of dichotomous outcome data from toe clip studies. Effect sizes calculated as risk ratio (RR) and corresponding 95% confidence interval (CI), using a random effects model. Right-hand side columns indicate events from total in treatment (clipped) and control groups. N.B.: a RR cannot be computed when there are zero events in both experimental groups. PND = postnatal day; h = hours post-clipping.

3.4. Quality assessment

The risk of bias and quality scores of the eight studies using separate control groups are shown in table 2 (individual scores) and figure 6 (overall scores). Although randomization of group allocation was mentioned in seven of these studies (87.5%; figure 6A), no study specified the method of

randomization (*e.g.* use of a random number table). Three out of eight studies (37.5%; figure 6A) reported that the experimenter performing the assessments was (partially) blinded to treatment, or that he/she assessed the allocation of the animals only after performing the outcome assessment. The other studies did not mention blinding of any phase of the experiment. None of the twelve studies included in this review reported a sample size or power calculation.

Because of the poor reporting of measures to reduce bias, the majority of items of the risk of bias tool were assessed as unclear risk of bias (figure 6B). Insufficient reporting of randomization and/or the method used led to an unclear risk of selection, performance and detection bias (items #1, 4 and 6). Baseline characteristics of the animals were adequately reported in two studies, in which we therefore assessed the risk of selection bias to be low (item #2). In all other studies, one or more baseline characteristics were not reported, leading to an unclear risk of bias. As regards blinding, we assessed the risk of performance bias (item #5) to be high in all studies, because ear and toe clipping are in practice impossible to conceal when performing the intervention, or when subsequently handling the animal. For this reason, we also assessed the risk of detection bias to be high in all studies (item #7), except for those in which the outcome assessors were not necessarily handling the animals. In the latter studies however, it was unclear if measures had been taken to adequately blind the outcome assessment, leading to an unclear risk of bias. Attrition bias (item #8) was assessed to be high in one study, where the numbers of animals allocated and included in the various outcome assessments could not be matched. Two studies scored correctly reported drop-outs, thereby scoring a low risk of attrition bias. In the remaining five studies, the risk of attrition bias was unclear. The risk of other types of bias was considered to be low in all studies (item #9).

Table 2 | Individual scores for study quality indicators and risk of bias assessment in eight included studies.

	Reporting			Risk of bias								
	Any randomization	Any blinding	Sample size calculation	Random Group allocation (selection)	Groups similar at baseline (selection)	Blinded group allocation (selection)	Random housing (performance)	Blinded intervention (performance)*	Random outcome ass.(detection)	Blinded outcome ass. (detection)	Reporting of drop-outs (attrition)	Other biases
Castelhano-Carlos2010	Y	Y	N	?	L	?	?	H	?	H	?	L
Cinelli2007	Y	N	N	?	?	?	?	H	?	?	L	L
Iwaki1989	Y	N	N	?	?	?	?	H	?	H	?	L
Miller2015	Y	Y	N	?	L	?	?	H	?	?	?	L
Paluch2014	Y	N	N	?	?	?	?	H	?	H	?	L
Rasid2012	N	N	N	?	?	?	?	H	?	?	?	L
Schaefer2010	Y	Y	N	?	?	?	?	H	?	H	H	L
Williams2008	Y	N	N	?	?	?	?	H	?	?	L	L

Y = yes; N = no; ? = unclear risk of bias; H = high risk of bias; L = low risk of bias; *blinding not possible.

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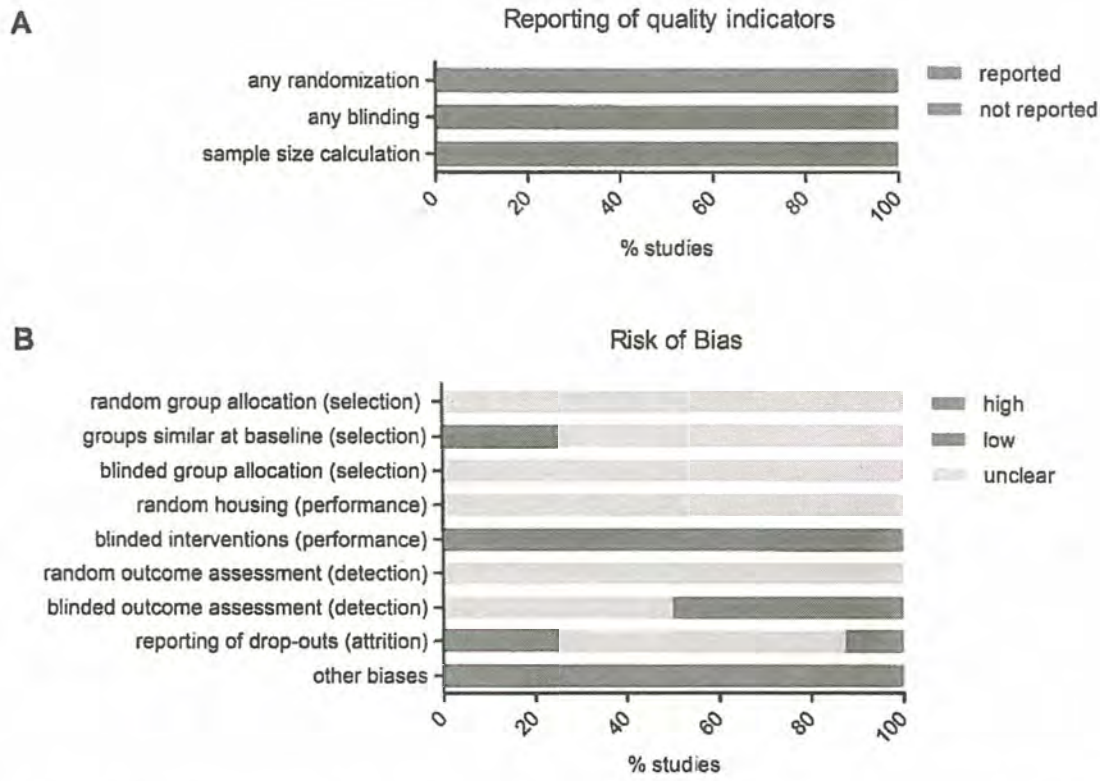

Fig 6|Risk of bias assessment and reporting of study quality indicators in eight included studies. A: Reporting of any mention of randomization, blinding or a power calculation. B: The risks of selection, performance, detection, attrition and other forms of bias were assessed using SYRCLE's risk of bias tool. Although randomization and blinding were mentioned in several articles, lack of reporting of the method used resulted in an unclear risk of bias for most items. Four studies were excluded from the assessment because their study designs are not compatible with the risk of bias tool.

4. Discussion

Because of their expected impact on animal welfare, toe or ear clipping are generally considered controversial techniques and their performance is restricted or even abolished in many animal laboratories. An abundance of guidelines is available on toe clipping and ear clipping, as well as other methods for individual identification and genotyping (e.g.[14-18]). However, these guidelines have, up to now, not been based on a systematic summary of all available evidence. Here, we provide the first systematic review of the evidence for the effect of toe clipping and ear clipping on rodent welfare.

4.1. Available evidence and quality

Studies investigating the effects of toe or ear clipping on rodent welfare are in short supply, and highly heterogeneous. This heterogeneity is mainly caused by differences in the population (males, females, various strains) and the intervention (age at time of clipping, number of sites clipped) under investigation, as well as the variety of outcome measures assessed. Most of the reported outcome measures showed no effect of toe or ear clipping on discomfort. Conversely, evidence indicating discomfort is present for ear clipping in the form of increased respiratory volume, vocalization and blood pressure, as well as for toe clipping, in the form of increased vocalization and decreased activity in pups, and reduced grip strength and swimming ability in adults. However, several limitations of the primary studies limit the reliability of both the evidence for and against an effect on discomfort, and hamper their interpretation.

Firstly, adequate reporting of methodological details in primary studies is crucial to determine the risk of bias in these studies and to assess the quality of a body of evidence. Our risk of bias assessment shows that the laboratory animal science field is no exception to the insufficient reporting of animal studies. We show that poor reporting of various aspects of experimental design resulted in most risk of bias items being assessed as unclear. This is a matter of concern, since evidence from preclinical animal studies indicates that lack of measures to reduce bias can severely

influence primary study results (e.g.[19]). A high risk of performance and/or detection bias is likely to be present in all included studies, and this should be taking into account when interpreting the results.

Specifying the primary outcome in a study prevents changing the primary outcome based on the study results, thereby reducing the risk of bias due to selective outcome reporting[20]. Unfortunately, none of the included studies defined which of the reported outcome measures was the primary outcome measure. In addition, none of the included studies reported a power calculation to support the number of animals used per group, even though this key element of experimental design is mandatory for approval by many animal ethics committees. The sample size calculation should specify the primary outcome measure, its expected mean and variation, and effect size the authors aim to detect. Doing so prevents the unethical use of animals due to overpowering (using more animals than necessary) or underpowering (using too few animals, especially relevant in cases where no effect of the intervention is found). Furthermore, knowing the planned sample size is often essential to assess the correct handling of drop-outs and attrition bias. At present, we are unable to assess whether any of the studies were adequately powered to detect differences between the groups in the outcomes under investigation.

In one study[10], the exact intervention applied in the control group was unclear, making it difficult to interpret the study results. In two other studies[3,9], the application of a secondary intervention in the control group may have introduced additional discomfort in these animals, thereby masking the effect of toe or ear clipping. One study[3] used a cross-over design, which can introduce carry-over effects that interfere with the intervention under investigation. In addition, outcome data are incompletely reported for many outcomes. We recognize that these inaccuracies are probably (partly) caused by the fact that some of the included studies were not specifically designed to assess the effect of toe clipping or ear clipping compared with handling or restraint. However, this indicates once again that studies specifically aimed to assess the effects of toe or ear clipping on welfare are very scarce.

As a result of these shortcomings, the effects of toe or ear clipping observed in a particular study cannot be directly generalized to other studies, or the population of laboratory rodents in general. Until more reliable evidence is available, an effect of toe clipping or ear clipping on animal welfare and study results can be neither excluded nor confirmed.

4.2. Implications for refinement

The current body of evidence is too small and heterogeneous to reliably assess the influence of, for example, species, age, sex, strain or clipping method, on the severity of discomfort. In three studies, outcome measures analysis was performed separately for male and female animals, but the observed effects did not differ between the sexes[9,11,12]. Schaeffer and colleagues found that pups undergoing toeclip at PND3 had a lower grip strength than pups clipped on PND7, which was attributed to the fact that the toes in three-day-old pups are partially fused together and too small to accurately clip the distal phalanx only, resulting in too much of the toe being removed[12]. Based on this finding, clipping would not be advisable before PND3, but replication of this result is needed to confirm this. Studies performing clipping on PND4[10] and PND5[9] reported that the procedure was quick and easy to apply, while our re-analysis of data from Paluch and colleagues suggests that clipping causes distress in seven-day-old pups (as indicated by increased vocalization and decreased activity), but not in seventeen-day-old pups. We conclude that the influence of age is presently unclear and further research is needed.

One study tested whether spray-on vapocoolant anesthesia could reduce pain during toe clipping, but concluded that the spray glued the toes together, which increased the risk of incorrectly clipping the distal phalanx of a single toe and increased distress due to prolonged handling. Furthermore, the vapocoolant interfered with haemostasis after clipping. Based on these results, application of this analgesic agent would not be advisable, but further research into suitable local anesthetics and analgesics may be worthwhile.

4.3. Implications for laboratory practice

The advantages and disadvantages of toe clipping, ear clipping, ear tagging and several other identification methods have been extensively described by specialist working / research groups, such as FELASA[14,18], the Norwegian Consensus Platform for Replacement, Reduction and Refinement of Animal Experiments (Norecopa)[15], and the joint BVAAWF/FRAME/RSPCA/UFAW working group[16]. In their 2008 report on toe clipping in mice, Norecopa reported that they had not been able to identify any studies providing electrophysiological or histological evidence on toe(tip) innervation in rodents that would allow for an assessment of the pups' ability to feel pain at the time of clipping. We did not identify such studies in our systematic search either.

Based on the available guidelines, there is international consensus that toe clipping should not be performed after PND7, since pups become increasingly active with age, which amplifies the risk of incorrect clipping and increases the level of restraint needed to correctly perform the procedure. This is independent of the observation that phalangeal ossification is complete around PND18, after which clipping is hypothesized to be more painful, although we found no data directly supporting this theory. In contrast, ear clipping is advised to be performed no sooner than PND14, due to the small size of the ears before PND14. Thus, toe clipping and ear clipping cannot be performed at the same age, and the age at which individual identification is needed is an essential factor in the choice of toe clipping, ear clipping or an alternative method for individual identification. Alternatives include tattooing or micro-chipping for identification, and hair biopsies or rectal swabs for DNA sampling (an overview of methods is provided in [14]). Most of these techniques cannot be used in newborn or very young animals and therefore cannot replace toe clipping. In addition, some of these techniques may cause more discomfort than toe or ear clipping[3,9]. Other factors influencing the choice of identification method is whether, and how much, DNA is required for quantitative or qualitative genotyping, and whether the identification should be permanent or temporary.

4.4. Directions for future research

The present review identifies a number of important shortcomings currently hampering the interpretation of the available evidence: 1) the low number of studies dedicated specifically to the assessment of discomfort after toe or ear clipping, 2) the lack of standardization of the chosen outcome measures, 3) insufficient reporting of experimental detail, especially regarding justification of the sample size and measures to reduce bias and 4) incomplete reporting of outcome data. Thus, further research is needed to provide reliable evidence on the effect of toe clipping or ear clipping on animal welfare. In order for future studies to succeed, all of these issues should be addressed.

First, future studies should be aimed specifically at assessing the effect of toe clipping or ear clipping on discomfort in laboratory rodents. Their design should include appropriate control groups, preferably one receiving no treatment (to provide a baseline) and one receiving sham treatment with handling and restraint only. No co-interventions should be applied apart from the intervention of interest. The animals should be handled and housed under the same circumstances. Furthermore, it is presently unclear whether characteristics such as species, strain, sex and age of the animals influence outcome, and future studies should be specifically designed and powered to reliably address these questions.

Secondly, upon submitting a proposal for new animal studies, researchers should provide rationale for their choice of outcome measures, including details on reproducibility and the optimal time point for outcome assessment. Ideally, the relevance and reproducibility of outcome measures used to assess discomfort and welfare in pups and adult mice and rats should be validated and discussed in the field, so that consensus may be reached and experiments may be standardized accordingly. We hypothesize that a multi-center approach may offer the opportunity to increase power and standardization future experiments.

Thirdly, we show that there is an urgent need to improve the reporting and methodological quality of (laboratory) animal studies. To this end, the ARRIVE guidelines[21] and the Gold Standard Publication Checklist (GSPC[22]) were published in 2010, but the reporting quality of studies included in this review was low regardless of the year of publication. Of note, the ARRIVE guidelines and GSPC do not

specify how detailed the reporting of measures to reduce bias should be, and SYRCLE's risk of bias tool[1] can provide guidance on how to report measures to reduce various forms of bias, in various stages of an animal experiment. This is especially important in studies on toe or ear clipping, since these procedures are very difficult to blind and the risk of biasing the study results is high unless adequate measures are taken. Reporting of a sample size calculation should be a mandatory for publication of future studies, especially since studies on toe or ear clipping need to be powered to reliably prove or disprove an effect on outcome. Finally, complete reporting of data for all outcome measures, either in the article, the supplementary material, or through open access data repositories is essential to reach reliable conclusions in future studies, for the benefit of science and animal welfare.

5. Acknowledgements

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Verzonden: donderdag 7 april 2016 11:46
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Onderwerp:

Beste

Ter kennisname. Is een motie die in het VAO Dierproeven van 22 maart jl. is ingediend, ging concreet over de teenknip, maar inhoud
motie is uiteindelijk breder geformuleerd.

De motie is aangehouden. De Staatssecretaris heeft gerefereerd aan de conclusies en resultaten die hij uiterlijk 1 juni a.s. van ZonMw krijgt over de
Teenknip. Daarom is naar mijn idee de motie ook aangehouden. Men wacht af. Maar, dit hangt dus als motie nog wel boven de markt.

Gr.

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Tweede Kamer der Staten-Generaal

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Vergaderjaar 2015–2016

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MOTIE VAN HET LID GRAUS
Voorgesteld 22 maart 2016

De Kamer,

gehoord de beraadslaging,

overwegende dat er voldoende alternatieven bestaan voor het merken van proefdieren ter vervanging van barbaarse methoden zoals het vaak onverdoofd couperen van tenen, oren en staart;

verzoekt de regering, het couperen van lichaamsdelen bij proefdieren (zolang deze nog bestaan) enkel toe te staan bij medische noodgevallen, zoals ter behandeling van trauma's door dierenartsen,

en gaat over tot de orde van de dag.

Graus

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Van:
Verzonden: donderdag 16 november 2017 10:12
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Hoi ,

Hierbij het definitieve document en de bijlagen.
Morgen komt zoals afgesproken de getekende aanbiedingsbrief ook digitaal en opnieuw het stuk officieel (nog gevolgd door met de post).

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Working Party Report

Report of the Federation of European Laboratory Animal Science Associations Working Group on animal identification

K Dahlborn[1], P Bugnon[2], T Nevalainen[3], M Raspa[4], P Verbost[5] and E Spangenberg[6]

[1]Department of Anatomy, Physiology and Biochemistry, Swedish University of Agricultural Sciences, Box 7011, SE-75007 Uppsala, Sweden; [2]Institute for Laboratory Animal Science, University Zürich, Winterthurerstrasse 190, CH 8057 Zürich, Switzerland; [3]Hevossaarentie 21, 37800 Akaa, Finland; [4]Consiglio Nazionale delle Ricerche (CNR), European Mouse Mutant Archive (EMMA), Campus "Adriano Buzzati-Traverso", Via E. Ramarini 32, I-00015 Monterotondo Scalo (Roma), Italy; [5]UMCN, University of Nijmegen, Geert Grooteplein Zuid 10, 6526 GA, Nijmegen, the Netherlands; [6]Department of Animal Environment and Health, Swedish University of Agricultural Sciences, Box 7068, SE-75007 Uppsala, Sweden

Corresponding author: K Dahlborn, Department of Anatomy, Physiology and Biochemistry, Box 7011, SLU, SE-75007 Uppsala, Sweden. Email: kristina.dahlborn@slu.se

Abstract

The primary aim of this report is to assist scientists in selecting more reliable/suitable identification (ID) methods for their studies. This is especially true for genetically altered (GA) animals where individual identification is strictly necessary to link samples, research design and genotype. The aim of this Federation of European Laboratory Animal Science Associations working group was to provide an update of the methods used to identify rodents in different situations and to assess their implications for animal welfare. ID procedures are an indispensable prerequisite for conducting good science but the degree of invasiveness differs between the different methods; therefore, one needs to make a good ethical evaluation of the method chosen. Based on the scientific literature the advantages and disadvantages of various methods have been presented comprehensively and this report is intended as a practical guide for researchers. New upcoming methods have been included next to the traditional techniques. Ideally, an ID method should provide reliable identification, be technically easy to apply and not inflict adverse effects on animals while taking into account the type of research. There is no gold standard method because each situation is unique; however, more studies are needed to better evaluate ID systems and the desirable introduction of new and modern approaches will need to be assessed by detailed scientific evaluation.

Keywords: Animal welfare, biopsy, refinement, rodent identification, toe clipping

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Rodent identification and animal welfare

The aim of this Federation of European Laboratory Animal Science Associations (FELASA) Working Group was to identify best practices for rodent identification in different situations and assess their implications for animal welfare. In general, the new EU Directive does not apply to practices undertaken for the primary purpose of identification of an animal (2010/63/EU). This means that it is left to the national authorities to decide what methods are acceptable or not. The overall aim of this report is to assist scientists in choosing the best identification method for their studies, and to enlighten legislators in the decision-making process.

Early individual identification is a prerequisite for valid and reliable research using animals; this is especially true in the case of genetically altered (GA) animals. Identification is necessary to link samples, research data and genotype to the individual animal. Loss of identification can render the animal unusable for further breeding and research. Data can also become unusable, which can result in compensatory use of additional animals. Reliable identification is therefore a prerequisite for good science and has important Reduction aspects. Although, identification methods are considered routine procedures, there is often a lack of scientific evidence related to their impacts on animal welfare and research outcome. Therefore, this report is based on best practice and, when available, on the scientific literature.

A wide variety of identification (ID) methods are used, each with different implications on animal welfare. Concomitantly, new and improved methods are being developed. The method of choice generally depends on tradition, study-based reasons and costs. A survey was performed in 2007 on the web-based forum Comparative

Medicine list (COMPMED), and it showed that the most commonly used methods are ear notching/clipping and ear tagging, both in the USA/Canada (ear notch/clip; 10 out of 23 answers, ear tag 11 out of 23) and in Europe (ear notch/clip; 10 out of 19 answers, ear tag 4 out of 19).[1] The least widely used methods were toe clipping and ear tattooing.

All identification methods are brief procedures, involving restraining the animals and result in some degree of discomfort and/or pain. Since these procedures are carried out on a vast number of animals, even minor improvements for the individual animal can lead to a considerable overall Refinement effect. A more expanded version, with practical details on how to perform different methods, will be published on the FELASA homepage.

Animal welfare is generally assessed with a combination of physiological and behavioural parameters. There are reliable parameters to be used for assessment of both acute and chronic effects on animal welfare. However, the identification procedure is often the first restraint to which animals will have been subjected, e.g. at weaning or even before, and therefore the assessment of acute effects of the identification procedure can be difficult to distinguish from the effects of the handling and/or restraint itself.[2,3] Reported long-term negative consequences of identification are increased mortality, systemic diseases, tissue irritation or damage, inflammation and tumours.[4–7] Therefore, a valid assessment of a specific identification method should include both immediate and chronic effects associated with the procedure. In addition, the ease with which the procedure can be performed, as well as the readability and sustainability of the marking over time, should be taken into account when evaluating the different methods. If genotyping is necessary, it can be considered as a true Refinement if the chosen identification method also generates a tissue sample, thus avoiding repeated (invasive) procedures on the same animal.

Acute effects are not only an animal welfare concern, but also a research quality concern. Invasive identification methods necessitate a recovery period before the animals can be used in the study. For example, the expected outcomes of tattooing are oedema and bleeding through puncture holes made by the tattooing instrument. This will temporarily affect the animals' physiology and behaviour which in turn may affect specific experimental parameters.

Analgesia and anaesthesia can be useful because they shorten the recovery period after invasive and painful procedures, e.g. surgery. The small size of the body parts used for identification makes it very difficult to apply local analgesics/anaesthetics. Inhalation anaesthesia must be considered in order to alleviate acute pain during the identification procedure. To eliminate postoperative pain, inhalation anaesthesia must be combined with analgesia. More studies are required to assess if the methodology could be refined to such a level that it would really alleviate the stress to which the animals are subjected during and after the identification procedure. In addition, the success of any method depends on the training and manual skills of the person performing the procedure and care of the instruments used.

In this report, the identification methods are grouped according to whether or not they are

(1) Permanent
(2) Invasive
(3) Generating tissue sample for genotyping.

Rodents are the largest group of mammalian species used in research and testing in the European Union (95%).[8] Therefore, the methods described here focus on rats and mice. However, most methods are also applicable to guineapigs, hamsters, gerbils and chinchillas.

Non-invasive temporary identification methods

Shaving or cutting the fur

Cutting the fur is one of the simplest methods for identifying laboratory rodents. An area on the body, mainly on the back (for visibility without handling), is cut or shaved. The most evident advantages are the ease of application and reading, along with the low cost. On the other hand, this type of ID can only be used to distinguish a limited number of animals.

The only necessary equipment is a fur shaving machine or a pair of scissors. The ease of reading the marking depends on the growth of the fur and the length of hairs that are removed. This method can be used after approximately two weeks of age, when pups have acquired a full coat of fur. The stress associated with this identification method mainly stems from the restraint of the animal. If properly performed, the procedure should not cause any pain to the animal.

Felt tip marker or alcohol-based pens for skin marking and coat dying

If one uses a felt-tip pen or similar markers, it is possible to identify mice or rats by writing marks or numbers on hairless parts of the body, e.g. ears and tail, but also on the skin (back skin, belly, limbs, armpits), especially in neonates or hairless mutants. The numbering or coding options can be increased by using different colours. The method is applicable to all ages, including neonates.

Commonly used dyes for colouring the fur are human hair colours or marking sprays meant for farm animals, but also a felt-tip pen can be used. Fur colouring is not suitable for a large number of animals. The marks are readable from a distance and without handling the animal. However, if neonates are marked on the armpits, handling is necessary in order to identify the animal. Coat dying is applicable as soon as the animals have fur, i.e. after approximately two weeks of age. Both skin marking and coat dying as such are painless but require restraint for application and renewing which can cause a temporary stress in the animal. This increased handling can also have positive effects.[9] These are low-cost but time-consuming methods, permitting identification of a limited number of animals only.

One major problem when using dyes or discolouring substances is their potential toxicity or untoward chemical

burden. These substances can enter the body by ingestion (e.g. grooming) or by diffusion through the skin which can lead to interference with research results.

Invasive temporary identification methods

Subcutaneous injection of ink

The subcutaneous injection of ink differs from tattooing because the ink is injected under the skin instead of into the skin layers. Like all subcutaneous depots of substances, ink also fades after some time (a few hours to a few days). The method provides limited numbering possibilities, although this can be increased by using different colours.

This procedure has two painful components; the insertion of the needle through the skin and thereafter the irritation due to the substance and/or the expansion of a body part as a result of the volume injected. In order to reduce the pain one should avoid using toxic or irritant substances. The footpad or the tail is the most common sites of injection. Leclerq and Rozenfeld reported a local swelling after footpad injection which may have been associated with pain.[10] This procedure requires restraining the animal and training the personnel. Handling is necessary to read markings on the tail and restraint is necessary to read the markings on the footpads.

This method can be used in animals of all ages, including newborns. It does however have a limited use since it only lasts for a short time (days at best). The Working Group does not recommend using this method since it is invasive and painful and only temporary.

Ear tag

Ear tags are available in metal or plastic; these are applied to the ear with special pliers. Tags are available in different sizes, to be used on different species, are pre-numbered and thus allow identification of a very large number of animals.

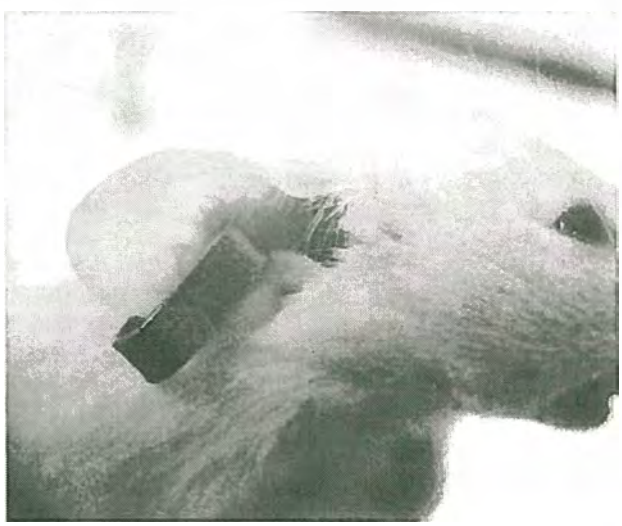

Figure 1 Tag placed correctly on the lower edge of the pinna

Figure 2 Tag placed in the upper edge of the pinna (incorrect)

The pliers are loaded with a tag and placed in the visible ear (*pinna*). Tags should be placed on the lower edge of the pinna so that they do not bend it (Figure 1). Ears should be checked regularly and in cases of tissue damage or inflammation, the tags need to be removed. Restraint is necessary for proper attachment of the tag and for its subsequent reading when identifying the animal.

This ID method is inexpensive and quick and easy to perform. There is some risk that the animal will lose the tag. The pinna is not developed before two weeks of age and to ensure that the ear is big enough to hold the tag, one need to wait until weaning. Furthermore, in experiments using magnetic resonance imaging (MRI) systems, the metal tags will need to be removed because they will interfere with the magnetic field. The method is painful and requires proper restraining of the animals and therefore they will temporary suffer for a brief period from a combination of pain and discomfort.

How the tag is placed in the pinna is important, with placement in the lower edge being recommended (Figure 1).[11] If the tag is placed on the dorsal side of the pinna, the ear might fold (Figure 2) and this could cause irritation and suffering to the animal.

Metal ear tags (commonly made from a nickel-copper alloy) have been associated with inflammatory and proliferative reactions and neoplasia in mice and rats following months of carrying the tags.[6,7] Similarly, a remarkably high incidence (8.8%) of squamous cell carcinomas has been reported.[4] The appearance of auricular chondritis, in C57BL/6 mice, was claimed to be an autoimmune response to reactive compounds released from the metal tag.[12] In that study, tagged ears contained higher levels of copper and cytokines, compared with non-tagged ears. When different tags were compared in guineapigs, the loss of tags decreased from 45% for metal tags down to 10% with nylon tags.[13] Hence, the risk of irritation and inflammation

seems to be related to the metal in the tags and to the duration for which the animals have to carry them.

Invasive permanent identification methods that do not yield a tissue sample for DNA analyses

Tattoo methods

Several body parts can be used for tattoo identification in rodents, e.g. ears, tail, footpads or toes. The procedure includes penetration of the skin with specific instruments in order to load tattoo ink or paste intradermally. The sensitivity of the body part where the marking is being applied will vary, and this has consequences on the extent of the pain sensation experienced by the animal in connection to the procedure.[14]

The ink must be loaded in the dermis, under the epidermis (the upper layer of the skin) to create a permanent marking. Hence, the skin barrier is disrupted and chemical compounds in the ink can spread via the circulation to the entire body. This poses two types of hazards: those associated with toxicity and those that may interfere with the study. Tattooing needles should always be clean (aseptic), kept sharp and replaced on a regular basis. Tattoo pigments are usually minerals, organic (industrial) or plastic-based pigments. Substances that are toxic or which may interfere with research results should not be used. In MRI studies, there is a specific problem with some tattoo inks.

Tattooing is a permanent method, but the ink may fade and become illegible with time. The number of possible unique identification marks possible varies depending on which method is used, but it can generally be increased by using different colours or by combining different locations. In general, tattooing procedures require training before they can be performed properly.

Ear tattoo

This method requires fully developed ears and a good restraint that fixes the head of the animal is necessary to avoid lateral movement and unnecessary tissue damage during the application. Restraint may be needed to read the tattoo.

One of the two methods for tattooing rodent ears is to use tailor made instruments, typically pliers with revolving heads. The second method available for tattooing rodent ears is the microtattoo system. This involves a pair of forceps with a disposable hypodermic needle on one side and a container with ink paste on the other, and the procedure is monitored through a magnifying glass (Figure 3). The identification consists of combinations of dots which allow for creation of a large numbering system. Generally, only handling is needed to read the ear tattoo.

Ears are considered to be very sensitive organs,[14] and hence tattooing must be considered to be at least moderately painful. Adult rats, instrumented with telemetric cardiovascular transmitters, were marked with three different methods; ear tattoo, ear notching and micro (toe) tattoo, using a crossover design. During the 1–4 h period and the following dark period, the mean arterial pressure was highest in the ear notching group indicating that the pain evoked was still present during 1–16 h after the marking procedure.[15]

Figure 3 Identification in ear with microtattoo equipment (photograph by Richard and Anne Boutet, Québec, Canada; reproduced with permission)

Tail tattoo

Tattooing on the tail can be performed in two different ways: the microtattoo system or the electric tattoo equipment (similar to that used in humans). Tail tattooing with the microtattoo system should only be applied in young animals before the ossification of the tail (tail ossification occurs between 2 and 3 weeks of age)[16] since it is inserted completely through the tail. Tail tattooing with an electric machine can be performed on adult mice, and rats of all ages if one wishes to write digits or letters. It can also be used in young mice to imprint dots or stripes on the tail. First the ink is applied on the skin and the tattoo needles transfer the ink into the skin layers and the remaining ink is then removed. This method allows for an infinite amount of numbers but needs some prior training in order to apply readable digits/letters. An alternative to the electric tattoo machine is the use of a lancet. It is done manually and produces only coloured dots, which results in a limited amount of numbers. As for the other tattoo methods, good restraint is necessary when tattooing the tail. In general, only handling is necessary to read the tattoo.

The pain, the necessity to restrain and the long duration of the tattooing procedure with the electric tattoo machine (noise, vibrations) cause discomfort to the animals. The manual method using the lancet also causes pain, the extent of which will obviously depend on the skill of the person performing the procedure.

Guillod and Johnson (1990)[17] found that ink tail tattoos caused mild fibrosis in tattooed areas and uptake of ink in regional lymph nodes, but no effects of tail tattooing on food consumption in a long-term study in adult rats. As a result of licking the tattoo, Sørensen *et al.*[18] found ink

Figure 4 Site of insertion (grey circle) of the needle (microtattoo) for toe marking

colour in the faeces of 20-day-old mouse pups after tail tattooing.

Toe tattoo or footpad tattoo

The microtattoo system can also be used to mark a toe or footpad by inserting the needle through the skin of these extremities. The needle should not be inserted through the whole toe or foot, only the pads are marked.

An important advantage of this method is the possibility to identify animals of all ages. Even when the toes of newborns are not yet separated, it is possible to perform toe tattooing. The microtattoo is inserted through the toe pad (Figure 4) or through the footpad.

A lancet can also be used to mark the pads of the toes or of the foot, employing the same method for tail tattoos and a lancet.

All toe or footpad tattoo methods described are considered to be painful, but the use of the microtattoo can be considered as acceptable because the needle used can be adapted to the size of the animal.

Microchip transponder

Electronic radio frequency ID transponders, commonly called microchips, are an effective way to identify laboratory animals. A microchip is inserted subcutaneously, in order for the animal to be identified with a transponder reader. The transponder responds to a low-energy radio signal emitted by a compatible reader, which displays the information (number) from the transponder. The microchip is implanted in the neck or further back, via a special syringe. The microchip system is a permanent ID method and allows for identification of an infinite number of animals.

Most readers can be connected to a computer which make it possible to collect a variety of data from a specific animal and transfer these directly to databases. Although the application is initially time consuming, the microchip has the advantage over other methods in that identification errors (except for incidental chip loss) are excluded. While it is

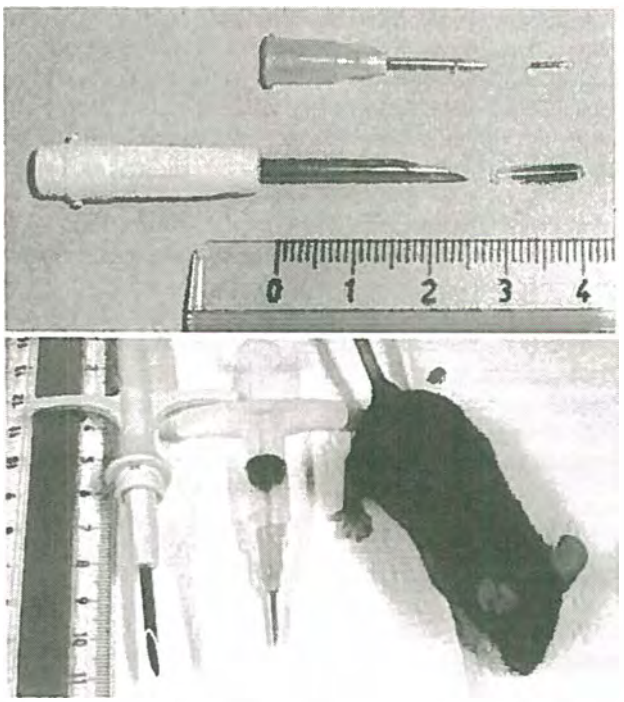

Figure 5 Size of microchips suited to mice (1 × 6 mm) and rats (2 × 12 mm) (top picture) and the size of the needles (12G, to the left and 18G, to the right) in relation to a fully grown C57BL mouse (bottom picture)

true that microchips can disappear, relocate or break, this risk appears to be relatively low. Rao and Edmondson (1990)[19] monitored 140 implanted mice for two years and found that 2% of the mice lost their chips, and 2.8% of the chips failed to transmit data.

The suitable time for microchip insertion depends on the size and body weight of the animal rather than age. The larger chip (12 × 2 mm) (Figure 5, top picture) is most suitable for animals ≥50 g and should not be used before adulthood in mice – if at all. The smaller chips (6 × 1 mm) are more suitable for mice (Figure 5, top picture). The manufacturer of the smallest microchip (6 × 1 mm) claims that it can be used in mice from five days of age, but according to Castelhano-Carlos *et al.*,[20] this is not recommended. Inhalation-anaesthesia is appropriate for the microchip implantation in all rodents.[21] It is recommended to close the wound, after insertion.

For proper application and correct positioning of the chip, some training is recommended. In general, handling, but not restraint, of the animal is sufficient to read the ID. For the small microchips, the reading distance is very short and thus it is necessary to touch the animal with the reader to collect the signal.

A recent study on five-day-old mouse pups showed that microchip (size 6 × 1 mm) injection resulted in a stronger reaction (sudden movements, urination and vocalization), compared with distal phalanx removal or toe tattooing performed at the same age.[20] None of the methods exhibited any postnatal effects. The authors recommended the use of toe clipping in young pups and microchips only after weaning.

In the long-term microchips can cause inflammation and fibrous tissue growth. Since they are implanted via injection, this is a procedure known to increase tumour risk. A causal link between microchips and cancer has been postulated to exist in rats and mice. Five out of eight articles reported that 0.8–4.1% of laboratory mice and rats developed malignant tumours around or adjacent to the implanted microchips.[22] In one of these eight studies, the investigators used a genetically modified line (p53+/− mice) that was prone to develop cancer, and 10.2% of the mice developed tumours[5] and in several cases these tumours also metastasized. The tumours generally occurred in the second year of the studies, in middle aged or older animals. However, in the Blanchard study, the heterozygous p53+/− mice developed fast-growing cancers before six months of age.[5] Taken together, microchip implantation appears to increase the risk of developing tumours. This is important to bear in mind when undertaking long-duration cancer research studies in rats or mice. One needs to consider also whether a foreign body like a microchip can affect immunological and skin studies (because of inflammation) or disturb image analysing techniques.

This is likely to be the most expensive identification method (costs for reader and transponders), and anaesthesia equipment may be necessary as well. Finally, the different types of transponders often transmit at different wavelengths and thus require different readers. This may need to be considered before transporting chipped animals between laboratories.

Invasive permanent methods of identification yielding tissue for DNA analyses

Ear notching

Ear notching as an identification method is generally considered to be easy to carry out and read and the necessary trauma to the animal is minimal in a properly executed procedure. The procedure can be applied on mice and rats. Punching or 'notching' holes in the ears requires a specific instrument. The choice of puncher is extremely important; there are punchers (mostly cheap ones), which pinch rather than cut and they should not be used.

The location of the holes must be accurate and done according to a chart/system in order to ensure a valid identification. The withdrawn tissue remnant(s) can be used for genotyping. The punchers must be completely cleaned between animals to avoid any DNA cross-contamination. The marking can be read from a distance but it may be necessary to pick up the animal from the cage. Metal ear punchers allow identification of the laboratory rodents by notching one or several holes in the pinna of the ear, at the edges of the pinna and/or in the middle. The numbering system of ear notching/punching allows identification of maximally a few hundred animals.[23]

In general, this can be considered a permanent method but there have been reports that the pinna can heal after several months depending on the size of the hole. Ear notching is painful and requires proper restraint of the animals and therefore they will briefly suffer from a combination of pain and discomfort. In order to read the ID it is necessary to pick up the animal, but generally restraint will not be needed. Training is required to learn the proper notching technique.

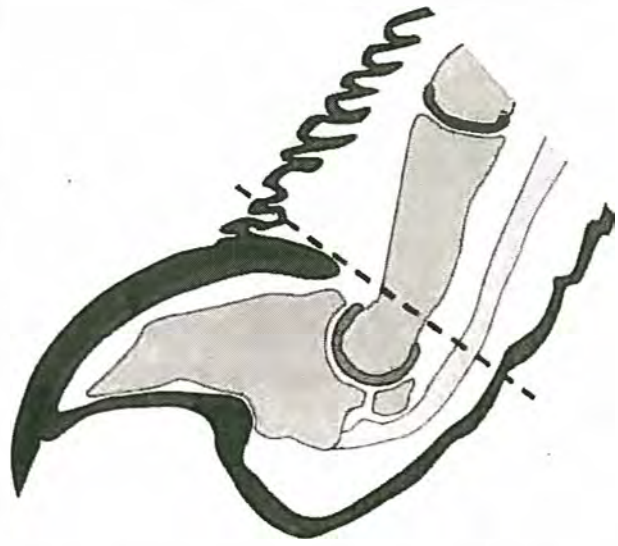

Figure 6 Schematic picture of the site of distal phalanx removal

Cinelli *et al.* (2007)[3] showed that different methods for biopsy collection in mice (tail biopsy, ear punch, mouth swab, rectum swab, hair collection) had the same effects on telemetrically recorded heart rate, motor activity and core body temperature as restraint alone. These parameters returned to normal levels one hour after the biopsy collection. In rats, during the first hour after the marking procedure, ear notching resulted in a higher blood pressure and heart rate responses than toe tattoo, which may simply be due to the fact that less restraint was needed for the latter procedure.[15] Using ultrasonic vocalization as a measure of pain, there was no difference in the response to ear notching and tail snip in mice.[24]

Distal phalanx removal

In distal phalanx removal, the entire distal phalanx of a toe is removed with sharp scissors from mouse pups around seven days of age. The cut is placed at the very distal part of the second phalanx (Figure 6) to remove the entire nail bed. In adult animals the identification is detected as a missing nail or tip of a toe on a paw. The method has been used in rats but is not recommended due to its long-term effects, e.g. impaired grip strength at weaning.[25] Therefore, the method will only be described for mice in this report. A similar, non-refined method generally known as 'toe clipping', where a larger part of the toe is removed, is sometimes used (because it makes the identification easier). However, the use of this non-refined method is strongly discouraged by the Working Group. National legislation within some European countries may actually prohibit the use of this method.

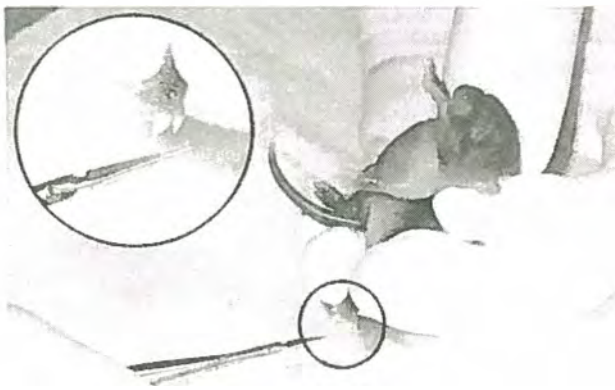

Figure 7 Restraining and cutting of the distal phalanx of a seven-day-old mouse pup (photograph by Dagmar Schaefer, Zürich, reproduced with permission)

The young pups are picked up and held by the nape of the neck in very much the same manner as the mother carries the pups. In addition, it is necessary to restrain the leg (Figure 7) to avoid sudden movements that might result in an incorrect cut. The scissors are opened and placed against the toe from below, in order to make it easier to see and cut the correct amount, which is 2–3 mm in a seven-day old pup (D Schaefer, personal communication). The removed phalanx biopsy can be used for DNA analysis (enough tissue for polymerase chain reaction).

The scissors used for this procedure should be small and sharp, e.g. ocular microsurgical scissors (Figure 7). The Working Group recommends a maximum of one toe per paw, i.e. four toes per animal, to be cut. However, since pups are marked while still in their native litter, males and females within the same litter can be given the same identification since they can be distinguished by sex. This can reduce the number of digits that need to be removed. The reading of the marking (a missing nail or tip of a toe on a paw) requires that the animal is picked up from the cage and held on a surface, such as the arm, to examine the paws. Sometimes restraint may be necessary.

This is a permanent method, if performed correctly, and it will not become illegible over time, which is a risk with some other permanent methods (e.g. tattooing and ear notching). The distal phalanx removal should only be performed on very young animals, Schaefer *et al.* (2010)[26] found that phalanx removal at day 7 was preferred over day 3 because at the younger age it was difficult to cut the correct amount since the toes were so small. Castelhano-Carlos *et al.*[20] successfully performed distal phalanx removal at five days of age and Spangenberg *et al.*[11] at six days of age. Already at 12 days of age the pups are very active and it becomes difficult to perform the phalanx removal with precision. An incomplete removal of the distal phalanx can lead to the regrowth of the digit.[27–30] It seems to be important to remove the entire nail bed to avoid regrowth. By around day 18, the phalanges have become ossified[31] which would make the procedure significantly more painful. Hence, the current knowledge shows that the age of 5–7 days (counting the day of birth as day 0) is a preferable time frame for removing phalanges for identification and genotyping of mice. There is no scientific support for successfully, and without inflicting more pain, performing the phalanx removal at later ages.

If the phalanx is removed correctly, the young pups display little or no reaction; mainly paw withdrawal, during or after the procedure.[20,26] The restraint mimics the mother's handling of the pups and might therefore be less stressful than the common techniques of restraining adult animals. There is usually a drop of blood on the cut tip but no further bleeding.[26] If further bleeding should occur, it can be stopped with a styptic or a haemostatic pencil. Schaefer *et al.*[26] found no effect of phalanx removal on grip strength nor did it cause hyperalgesia (tested at 12 weeks of age) when phalanges had been removed on day 7, but grip strength was impaired when the phalanges were removed on day 3. It is likely that too much of the toe was cut on day 3 due to the small size.[26] No effects on climbing abilities at weaning,[11] or as adults,[20] were found in mice with distal phalanx removal.

Today this method is in fact the only permanent identification of a pup with a simultaneous tissue sampling for genotyping. Because of the early genotyping, a rapid and early selection of animals needed for studies or further breeding is possible which eliminates the costs of housing redundant animals. The recommendation of the Working Group is to use this method only in combination with genotyping and exclusively for young pups. Other methods are options only for marking to only mark young animals when no biopsy is needed.

New methods

The first method described below has recently become commercially available (2010) and is a modification of the classical ear tag. The second method is also commercial available. The final two methods described use completely new approaches to identifying rodents. However, they are not yet commercially available.

Mini-ID Ear tags (http://www.zonotid.com/)

A recent alternative to traditional ear tags is a lightweight plastic tag (0.07 g) that has a 2D barcode etched onto a titanium plate attached to the plastic tag. It is read using a barcode reader similar to the microchips. Its application to the pinna is similar to that done with the traditional tags. This tag type is lighter and should therefore reduce the risks of infections or inflammation in the ear caused by irritation from a heavy tag. It does not have the loop shape of a metal ear tag which minimizes the risk of it becoming entangled. Further, the plastic material reduces the risk of an allergic reaction to the tag. Like other ear tags, this

Table 1 Overview of available identification methods for rodents; issues related to both techniques and the animals

	Concerning the technique							Concerning the animal	
Identification method	Permanent/ Temporary	Specific skills/ training	Number of codes	Age for application (from)	Anaesthesia*	Aseptic measures†	Sample for DNA	Pain/ Discomfort at application	Handling/ restraint at reading
Shaving/cutting the fur	T	No	5	2 weeks	No	No	No	D	None
Skin marking	T	No	10/colour	All	No	No	No	D	None/H
Coat dying	T	No	5/colour	2 weeks	No	No	No	D	None
Subcutaneous ink injections	T	Yes	~8/colour	All	No	Yes	No	P	H/R
Ear tag	T	Yes	Infinite	Weaning	No	Preferable	No	P	R
Ear tattoo	P	Yes	Hundreds	Weaning	No	Yes	No	P	H/R
Tail tattoo	P	Yes	Hundreds	Weaning	No	Yes	No	P	H
Tail microtattoo	P	Yes	Infinite	~2 weeks	No	Yes	No	P	H
Toe/foot pad tattoo	P	Yes	Hundreds	All	No	Yes	No	P	R
Microchip transponder	P	Yes	Infinite	Depends on body size	Yes	Yes	No	P	H/R
Ear notching/ punching	P/T	Yes	Hundreds	2 weeks	No	Preferable	Yes	P	H
Distal phalange removal	P	Yes	Hundreds	4–8 days	No	Preferable	Yes	P	H

For detailed information see corresponding section in the report
The first three methods mentioned in the table are non-invasive
*Analgesia explained in welfare paragraph
†Depending on body part, see tattoo paragraph

method can be used from weaning and allows identification of an infinite number of animals. Other types of ear tags are also becoming available on the market.

Microtransponder p-Chip

This is a new method with a radiofrequency identification tagging that uses 500-μm, light-activated microtransponders implanted subcutaneously into the ear or tail of mice. According to Gruda *et al.* (2010)[32] the preferred location for implanting is in the side of the tail, because implantation at this site was reported to be simple to perform and was associated with shorter implantation times and a higher success rate compared with the ear. They claim that the main benefits of using light-activated microtransponders over other identification methods are their small size which minimizes stress to the animals during implantation.

A biometric approach to laboratory rodent identification

This new technique uses the blood vessel pattern of the pinna of an animal as biometric identification. Each animal has an individual blood vessel pattern, like a fingerprint. Images of the ears of all animals that are to be identified are taken. Later when an animal has to be identified, a new picture is taken of the ear and this is compared with the information stored in the database to identify that individual.[33] It can likely be used from weaning, when the ears are large enough. Since the blood vessel patterns are unique for each individual, this technique should allow identification of an infinite number of animals.

Luminescent Micro Tattooing LMT

With this method, very small luminescent pigments are applied to the skin of the animal, such as on the tail base or the ear, in the form of dot writing (like Braille). It is called 'Luminescent Micro Tattooing LMT'. It applies the code by means of micro-needle arrays coded with pigments for one-time use. Each array represents exactly one code, as in Braille. The code is applied simply by putting manual pressure on all needles. The needles penetrate the skin of the animal, but only certain needles contain pigment, thus producing the code on the skin of the animal. The dot writing (data matrix code) can be read and decoded in a scanner.

Conclusions and recommendations

The working group strongly believes that it is important that the choice of identification method is based on scientific evidence rather than personal opinions and local traditions. Undoubtedly, more studies are needed to thoroughly evaluate identification methods and the introduction of a new method should be preceded by detailed scientific evaluation.

According to the Guide for the Care and Use of Laboratory Animals (1996) 'toe clipping as a method of identification should be used only when no other individual identification method is feasible and should be performed only on altricial neonates'.[34] When the Working group of Rodent identification began its work, distal phalanx removal was identified as a method with potential problems and therefore recommended as an area for further research.

Spangenberg *et al.*[11] initiated and performed one study and the study by Castelhano-Carlos *et al.*[20] was also performed after contact with members of the working group. In addition a third study has recently been published.[26] In summary, these recent studies have demonstrated that distal phalanx removal does not have any negative effects on growth and physical and behavioural development in young mouse pups.[11,20,26]

General recommendations:

- The ideal identification method should provide reliable individual identification, have no adverse effects on the animal or the animal model and be technically easy to apply;
- The choice of method depends on the age and size of the animal, whether or not a tissue sample is necessary, whether every animal needs a unique number, the duration of the study and whether the identification method can interfere with the research results or their interpretation;
- We recommend that proven permanent methods should be used for long-term identification and non-invasive temporary methods should be used when appropriate;
- Key points to consider while choosing an ID-method are summarized in Table 1.

If unique numbers are needed for every animal, tail tattooing, (non-metal) ear tags or microchips are the only methods possible. For long-term studies (> 3 months), tail tattooing is the safest method of these, since ear tags may be lost and microchips might induce tumours. Metal ear tags are the worst choice of an ID method because of the associated pain and distress as well as the risk of ear infections, tumours and allergic reactions. Moreover, subcutaneous ink injection is an invasive, painful but non-permanent method and is therefore not recommended as a method of identification.

All the new methods have advantages in terms of animal welfare and are recommended although their availability (and applicability) may still be limited. It is hoped that scientific studies will be conducted comparing these new methods with the most commonly used methods available today. So far, the microchip is the only method available supporting 'online' data transfer to a computer. It is notable that all four new identification methods described do provide a computer ID as well.

An important area for future research is the use of analgesia/anaesthesia during and after identification procedures. Inhalation anaesthesia does not in general alleviate post-operative pain. Therefore a special treatment for pain alleviation should be combined with this anaesthetic method. However, long-term pain is likely to have a more adverse effect on animal welfare than any acute pain experienced during and identification procedure. Local analgesia could be applied but this need to be administered in advance before it will have any effect. However, the body parts where it needs to be applied are very small and the animals can lick off any ointment used. Sometimes anaesthetics are aversive, which means that the degree of aversion from the anaesthetic could actually be greater than the transient pain of, e.g. ear notching (P Flecknell, personal communication). In addition, the restraining necessary for performing identification procedures has been shown to be as aversive to the animals as the identification procedure itself.[3]

In summary, the choice of identification method should be determined so as to minimize the adverse effects on the animals, while at the same time taking into consideration the type of research to which the animals will be subjected. There is no gold standard method because each situation is different (see above). Here too, good science and animal welfare go hand in hand.

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Norecopa
c/o National Veterinary Institute
P.O. Box 750 Sentrum
0106 Oslo
Norway
www.norecopa.no
Correspondence to: Adrian Smith (adrian.smith@vetinst.no)
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Toe clipping in mice: an evaluation of the method and alternatives

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1. Background

1. On 2 May 2008 the Norwegian Animal Research Authority (Forsøksdyrutvalget, FDU) forwarded to the Norwegian Food Safety Authority (Mattilsynet, MT) a complaint made about their decision of 26 February 2008 to allow toe clipping as a method for the identification and genotyping of genetically modified mice. FDU had set the following conditions for their decision:
    a. toe clipping must be performed before the age of 10 days
    b. only one toe on each hindleg must be clipped
    c. the toe must be anaesthetised with a local anaesthetic (gel or dip method) before clipping
    d. Clipping must be performed before bone tissue and periosteum have been formed.

    In the application for animal research upon which FDU made their decision, it was specified that clipping was to be performed no nearer the body than the first toe joint. The method should therefore be referred to as ***toetip clipping***. FDU considered other methods such as ear marking to be equally severe procedures, at least. FDU were aware of studies on the effect of toe clipping at the University of Zurich and will compare these findings with the results from studies on other marking methods. Based on available knowledge and experience, FDU concluded that they could not see that toe clipping, under the conditions described above, is particularly burdensome compared with other identification techniques.
2. In a letter to Norecopa dated 1 January 2008, FDU requested an evaluation of the different strategies for toe clipping rodents (time for clipping, how far on to the toe should be clipped, the number of feet where toes should be clipped, the number of toes that should be clipped per foot) in relation to other relevant methods for marking and tissue sampling, as regards both animal protection and ease of use. On 11 August 2008 Norecopa replied that it was willing to undertake the task. An early draft of its conclusions were discussed at Norecopa's board meeting on 17 September 2008.
3. In a letter dated 24 September 2008, MT accepted the complaint lodged against toe clipping and reversed the decision made by FDU. MT pointed out that removal of a body part is against the principles embodied in the Norwegian Animal Protection Act, that animals should be given the benefit of the doubt, and that scientific studies are underway to evaluate the methods available. MT questioned the claim that neonatal animals do not feel pain. Until the results of ongoing studies are available, MT wished to be restrictive.

2. Introduction

Reliable and repeatable identification of individuals with a minimum of stress to the animal is an elementary and necessary factor in the quality assurance of most animal experiments. The increasing use of genetically modified animals in laboratory studies, where large numbers of animals are brev in the hope of producing individuals with the desired genotype, has created a need for individual marking and tissues-sampling of as young animals as possible, for

practical and economic reasons. Early humane killing of surplus animals can be claimed to have ethical advantages, because it eliminates the risk of suffering in individuals which will not be used in experiments anyway.

There are few published studies on methods that can be used for tissue sampling in young genetically modified animals, and even fewer behavioural studies of the effects which these methods have on the animals in the longer term.

It is important to differentiate between quantitative and qualitative genotyping. When new transgenic mouse lines are established, it is often desitable to be able to measure quantitatively how the gene of interest has been incorporated into the genome (the number of copies and location). For these measurements a method like Southern blotting is used. In established lines, it is usually sufficient to detect the gene qualitatively (to see if the animal is transgenic or not) and in these cases methods such as PCR, which are based on the amplification of DNA from very small samples, can be used. In other words, the aim of the tissue typing decides how much tissue one needs to take from the animal. A range of relatively non-invasive methods for DNA-sampling for PCR testing have been developed. In the case of quantitative tissue-typing, however, invasive methods have to be used, that in reality consist of amputations: tail clipping and toe clipping.

The decisions made in research communities in Norway and abroad on the choice of method appear largely to have been made without a solid base of comprehensive and complete scientific studies, and are to a large degree a result of the researchers' subjective opinions or asssumptions, based in turn on extrapolations from human experiences. A review of the literature reveals that descriptions of toe clipping are more positive in older publications and guidelines. Norecopa has not found histological or electrophysiological studies of the innervation of the toes in rodents, that could provide an anatomical or physiological basis for assessment of the animal's ability to experience pain when toetip-clipping is performed at the age in question.

It is a generally recandnised concept in modern laboratory animal science that choice of method should be based on an evaluation of several factors:

- the method's scientific quality and reproducibility
- the effect which the method has on the individual on which it is used

Both these two factors should be prioritised before practical and economic considerations.

In Norecopa's opinion there is a need for similar evaluations of the methods used for identifying and tissue-typing wildlife (http://www.nc3rs.org.uk/category.asp?catID=79) and fish (http://oslovet.veths.no/gardermoen.pdf) in research.

3. Terms in use

The Norwegian Regulation on Animal Experimentation §2 gives guidelines for what can be defined as 'simple marking of animals'. Methods for marking animals are not defined as animal experiments as long as there is no 'reason to assume that the experiment will affect the animal's normal way of life, or cause other than slight pain or discomfort of a highly temporary nature'. This type of division into regulated and non-regulated procedures exists in a number of other countries, such as Great Britain:

'Blood or DNA sampling solely to establish the identity or provenance of an animal would not be regulated if the intervention caused no more than momentary discomfort or distress.

Methods of marking or identification, such as toe clipping, which can cause suffering in excess of this threshold, are regulated when carried out for an experimental or other scientific purpose. (www.nc3rs.org.uk)

Toe clipping has been commented upon in the mass media in Norway, in these articles, among others:

1. Researchers want to mutilate mice (Norecopa's translation) (www.nrk.no/nyheter/distrikt/ostlandssendingen/1.5879860).
2. A letter to the editor of Aftenposten 10.06.08 from Bodil Ekerhovd Damsgaard, Oslo, who claims that toe clipping reduces the animal's ability to climb and groom itself.
3. A letter to the editor of Aftenposten 14.06.08 from Janicke Nordgreen and Tórunn Fosse, Norwegian School of Veterinary Science, who point out that it is the degree of development of the nervous system that determines whether or not a newborn mouse can feel pain, not the size of the sample taken or the degree of skeletal ossification. The authors also point out that research indicates that the body's mechanisms for reducing painful stimuli are probably not fully developed at birth, such that stimuli may be more painful to newborn animals than they are to adults. In addition, pain experienced in the neonatal period may be "remembered" by the central nervous system, so that the animals show increased sensitivity to pain later in life.

These articles in the media do not always give a precise description of the method for which FDU gave permission, see section 1.1 above.

4. Methods of identification

In this section, the advantages and disadvantages of the different methods for identification and tissue-sampling are briefly described.

1. Toe clipping

Advantages:

- It is a permanent identification method with a low risk of misidentifying individuals (Kumar, 1979)
- It provides enough DNA for quantitative genotyping
- It allows the identification of desirable genotypes before weaning (Nadon & Draeger, 1996)
- It has been reported that the tip of a toe on the forelimb of a mouse will grow out again if the amputation is performed distal to the outermost joint (Borgens, 1982)
- The method is simple and cheap. It must, however, be performed carefully if only the tissue distal to the outermost joint is to be removed
- The method requires minimal restraint of the animal (the animal's hindquarters are lifted up by gripping the base of the tail, or the animal is lifted by the skin of its neck in the same way as young are transported by their dams)
- Local anaesthesia of the foot can easily be carried out using a spray
- Inspection of the toeclip is quick and easy, with small chances for misidentifying two animals

Disadvantages:

- There is reason to believe that the method is painful, regardless of whether the toe contains bone or cartilage at the time, and the animal will experience pain after the procedure, even if analgesics or anaesthetics are used
- There is some behavioural evidence of reduced welfare after toe clipping (Iwaki *et al.*, 1989)
- It may be assumed that mice use all their toes for normal locomotion, including climbing, and that some degree of locomotory impediment arises after toe clipping. Mice in cages appear, however, primarily to use their forelimbs when climbing. The removal of one toetip on one hindleg will probably have limited effect on their ability to climb. On the other hand, the toe stump will be in more or less continuous contact with the cage floor after the procedure, unlike the situation if tail clipping is performed

2. Ear punching

Advantages:

- The method is similar to earmarking used in farm animals, which is a standard practice and therefore generally accepted
- The method is considered to cause less discomfort than tail or toe clipping, because it is performed in an area without bone formation
- The technique provides tissue for qualitative genotyping
- The size of the hole kan be reduced to 0.5 mm and still give enough tissue for PCR (Hawkins *et al.*, 2006)

Disadvantages:

- The method is likely to be painful, even though the ear contains cartilage and not bone. The animal will probably experience pain after the procedure, even if painkillers are used during the procedure
- The method cannot be used for quantitative genotyping
- Problems with reading the ear punches are common, particularly if there has been fighting in the cage, since some management systems require the use of more than one hole per ear. Animals can easily be confused using this technique, which may in turn lead to the use of more animals in a study
- The ear that has been punched may become the target of aggressive behaviour in the cage and the ear may be torn into pieces
- Ear punching requires considerable restraint of the animal, since the operator will want to avoid being bitten in the process
- Animals cannot be ear punched before they are 14 days of age, since the ears are too small to be marked without causing extensive damage to the ear
- The ear is given a permanent perforation, which reduces its thermoregulatory function and the ability to localise sound. The outer ear is an important organ in the mouse for the reduction of body temperature following exertion, since these animals cannot lose water vapour by gasping or sweating

- Local anaesthesia of the ear is difficult because of its proximity to the ear canal and eye

3. Earmarking with metal tags

Advantages:

- The method is the equivalent of earmarking domestic animals, and is therefore generally accepted
- The method is likely to cause less discomfort to the animal than toe clipping as an identification technique, because it takes place in an area without ossification
- It is an unequivocal method of identification

Disadvantages:

- The method is considered to be painful, even though the ear contains cartilage and not bone. The animal will probably experience pain after the procedure, even if painkillers are used during the procedure itself
- Ear tags have to be placed where the cartilage is thickest. This necessitates long experience if they are to be placed correctly in mice
- For this reason, ear tags frequently fall out. If this happens on two animals in the same cage, both must be marked a second time
- Ear tags can result in the ear being caught on cage furniture
- Ear tags can become a target for aggressive behaviour in a cage, with the risk of them being ripped out
- This method does not provide tissue for genotyping
- Animals cannot be tagged before they are 14 days of age, because the ears are not big enough
- The method requires considerable restraint of the animal
- The weight of the tag results in the ear hanging in a lower position than normal, which reduces its function in thermoregulation and the localisation of sound
- Anaesthesia er difficult because of the proximity to the ear canal and eye

4. Tail clipping

Advantages:

- The method provides enough DNA for quantitative genotyping
- It enables animals with a genotype of interest to be identified before weaning
- The method er simple and cheap
- Experience indicates that the method er less painful than ear punching if no more than 5 mm of the tail are removed
- The method is quick and does not require a form of restraint that stresses the animal

Disadvantages:

- The method is likely to cause pain, regardless of whether the tail contains bone or cartilage, and the animal will probably experience pain after the procedure. It is possible that the pain during and after the procedure can be reduced by using

anaesthesia, and this should be investigated more closely. It must also be assumed that suffering after the procedure can be reduced by treatment with painkillers.

- The tail is an important organ for balanse and grasping, and a degree of locomotory impairment will arise after this procedure if significant lengths of the tail are removed, for example by serial sectioning
- Removal of only the very tip of the tail will on the other hand have minimal effects on locomotion
- There is a danger of bleeding if the technique is used on older animals. Repeated tail sectioning is therefore not recommended
- The method must be combined with a means of identification

5. Tattooing

Advantages:

- It is a permanent method of identification with little risk of misidentifying individuals
- The method can be used on very young individuals, for example on the palm of the paw (Honma *et al.*, 1986)

Disadvantages:

- The method is difficult to use on animals with pigmented skin
- It does not provide tissue for genotyping
- The effect of tattooing on the toes and palms of small rodents has not been sufficiently investigated
- Tattooing is experienced as painful in humans and the same must be assumed for rodents
- The method can result in activation of the immune system due to uptake and storage of dye particles in tissue macrophages. This is an undesirable factor in many animal experiments

6. ID-chip (transponder)

Advantages:

- This is a permanent method of identification with no risk of misidentifying individuals
- Smaller transponders have been developed in recent years (e.g. Nonatec, www.nonatec.net) which may be assumed to cause a minimum of discomfort. These can be used on young animals
- The transponder is implanted under the skin of the neck, which is an area where the animals are grasped by their dams when they are moved around the cage. It may therefore be assumed that this procedure causes a minimum of fear

Disadvantages:

- The method does not provide tissue for genotyping
- The method requires equipment, including a scanner that supports the system used in the transponder. Differences between systems can cause problems when animals are moved from one laboratory to another

- The larger transponders are likely to be burdensome for small individuals. The needles used to implant the transponder are large and analgesia should be used during implantation

- There is somem evidence of an increased frequency of cancer in rodents that have been implanted with transponders (http://en.wikipedia.org/wiki/VeriChip)
- The transponders can cease to function after a while
- The transponders can wander under the skin, making it difficult or impossible to scan them

7. Blood sampling

Advantages:

- The method does not involve the removal of part of the animal's exterior organs

Disadvantages:

- Blood sampling in small animals is technically demanding and can result in pain and discomfort
- The method must be combined with a means of identification
- The method provides little tissue for genotyping and is most suitable for research where groups of animals are to be separated based on the phenotype of their blood cells
- The method requires some equipment and experienced operators

8. Application of dye to the skin, hair clipping etc.

Advantages:

- There is little risk of misidentifying individuals if the operators use a standard system
- The methods are animal-friendly, simple and cheap

Disadvantages:

- The methods do not provide tissue for genotyping
- Dyes can be rubbed off and frequent handling is necessary to apply more dye or to clip the hair
- In toxicological studies, the use of chemicals that can penetrate the skin should be avoided
- The chemicals may affect the other animals in the cage if they lick each other

9. Saliva/epithelial cells from the buccal cavity

Advantages:

- The method er animal-friendly in individuals that are sufficiently large (Irwin *et al.*, 1996).
- The method provides DNA for qualitative genotyping (Zhang *et al.*, 2006)
- Extraction of DNA from the sample is faster than when using ear punching, tail clipping or toe clipping (Meldgaard *et al.*, 2004; Mitrecić *et al.*, 2008)

Disadvantages:

- The method can only be used for qualitative genotyping (PCR)
- The method must be combined with a means of identification
- There is a risk of contamination by DNA from the mother's milk or mammary glands
- The method is burdensome for young mice
- There is a risk of contamination between animals because they lick each other and consume each others' faeces. The method is therefore not suitable when several animals are housed in the same cage

10. Rectal scrapings/faecal samples

Advantages:

- The methods are animal-friendly and cheap
- Extraction of DNA from the samples is faster than with ear punching or ear/toe clipping (Murgatroyd *et al.*, 2006)

Disadvantages:

- The methods must be combined with a means of identification
- The chance of contamination of faecal samples, or confusion between animals, is great, unless the samples are collected directly from the individual, which is a time-consuming process
- The samples contain DNA from a range of other sources than the animal itself
- Rectal scrapings cannot be performed on very young animals
- The method has not been used extensively, particularly on young animals

11. Removal of hair follicles

Advantages:

- The method er relatively animal-friendly and cheap
- Hair plucking requires minimal restraint

Disadvantages:

- The method must be combined with a means of identification
- Mouse hair easily becomes charged with static electricity, which can result in contamination between individual, either due to hair moulted from other animals or because of insufficient cleaning of equipment between samples. The method is, however, suitable for sampling just a few animals, for example in cases of repeated genotyping
- The method er unsuitable for small animals that have little hair

12. Earprint

This is a new method, inspired by the use of fingerprinting in humans, based upon photography and interpretation of the pattern of blood vessels in the ear of rodents (Cameron *et al.*, http://www.nc3rs.org.uk/news.asp?id=675).

Advantages:

- The method er animal-friendly, rapid and non-invasive

Disadvantages:

- The method is still under development and requires special equipment
- Some readings are of insufficient quality to enable definite conclusions to be reached and the animal has to be re-photographed
- The method does not provide DNA for genotyping

5. Research on toe clipping and other methods

Little research has been published on toe clipping.

1. Vachon (1998) studied the anatomical changes following amputation of the distal end of the first bone at approx. 2 weeks of age. Complete healing of the bone and the overlying skin was observed, but changes in the normal architecture of the bones were observed. Ossification occurred on day 18. No problems were observed after amputation but the author pointed out the need for studies of the innervation of the area and the possible risk of inflammatory reactions.
2. Cinelli *et al.* (2007) did not evaluate toe clipping, but they compared the effects of many biopsy techniques using telemetry of body temperature and heart rate, and locomotory patterns. They concluded that restraint was the most important stress factor for the animals and that hair samples were difficult to handle because of the risk of cross-contamination. Ear marking was the method that gave the greatest and most longlasting effects on the parameters they measured, but all the same it was considerd to be the best method since it functioned both as an identification technique and a biopsy method. The authors pointed out that buccal and rectal scraping often resulted in bleeding and could therefore not be reckoned as non-invasive methods, neither did they give a different stress response than the other methods investigated.
3. Arras *et al.* (2007) studied the effect of tail clipping with or without anaesthesia on a range of physiological parameters in adult mice. They concluded that anaesthesia did not reduce the effects of tail clipping on a range of physiological parameters, and that tail clipping affected these parameters less and for a shorter period than did anaesthesia alone. In a short description of the histology of the tail, they reported that there was little difference between sections taken 2, 6 and 10 mm fra the tip, except for a gradual reduction in the number of nerve fibres further away from the body.
4. Hankenson *et al.* (2008) have recently published a study of ossification, DNA-content and acute behavioural response to tail clipping (various lengths) in 6 mouse lines between 3 and 42 days of age. The authors concluded that the optimal time for harvesting DNA was 14-17 days of age.
5. The European laboratory animal science organisation FELASA (www.felasa.eu) has appointed a working group that is comparing the various methods for identification of rodents. The first phase of their work is a pure literature study. Their conclusions are not available yet.
6. FELASA has also appointed a working group to investigate the possibilities that exist for refining the methods used to genotype genetically modified rodents. The deadline for this report is December 2009.

7. A research group in Utrecht is in the process of comparing tattooing with toe clipping in newborn rodents.
8. Researchers in Uppsala are working on a comparison of toe clipping and earmarking in various mouse strains.

The conclusion that must be drawn is that a number of studies are in progress, but at the present time there are few results of scientific experiments that are of relevance to the current topic.

6. Important considerations when choosing a technique

1. Norecopa is of the opinion that both scientific, legal and ethical considerations should for the basis of judgement when a procedure to be used on an experimental animal is evaluated. As an aid to this process, the "3 R's" of Russell & Burch (1959) and the "3 S's" of Carol Newman (cited in Öbrink & Waller, 1996) may be used:

   Replace, Reduce, Refine

   Good Science, Good Sense, Good Sensibility
2. Any pain or suffering involved is experienced by the individual, regardless of the number of animals used.
3. The total burden placed on the animal in its lifespan should be considered. If a procedure carried out at one phase can eliminate the need for further harmful procedures later, then it should be considered, even if in itself it is a burden to the animal.
4. The advantages of tranquillisers/sedatives or anaesthetics must be weighed against the stress imposed by the handling necessary to administer these drugs, or the drugs themselves. This stress may be greater then the stress of the procedure itself, but optimal use of tranquillisers/sedatives can also contribute to a reduction of the burden on the animals. This must be assessed in each specific case. The Norwegian Regulation on Animal Experimentation §14 states that 'should there not be reason to assume that the intensity of pain experienced in an experiment exceeds the pain intensity of anaesthesia, anaesthesia may be omitted'.
5. The aesthetic aspect of the amputation of parts of a body organ is important for many people, and traditional methods such as earmarking are more generally accepted in general opinion.
6. The Norwegian Animal Protection Act is in general restrictive to amputations. The Act forbids tail docking, beak clipping of chickens and ear cropping of dogs. The present Act is to be replaced in the near future by a new Animal Welfare Act. The restrictive attitude to amputations is present in the draft of the new Act. Although the Norwegian Regulation on Animal Experimentation allows procedures to be performed that are normally forbidden in society's use of animals, in Norecopa's opinion the research community should be especially cautious in employing techniques that fall into that category.
7. Toe clipping is in Norecopa's opinion within the category of identification and sampling techniques where, according to the Norwegian Regulation on Animal Experimentation (§2) 'there is reason to assume that the experiment will affect the

animal's normal way of life, or cause other than slight pain or discomfort of a highly temporary nature.'

8. Toes are more or less in contact with the ground at all times, in contrast to other organs under discussion, such as the tail and ears.
9. A central factor in the evaluation of toe clipping is whether there is a real need for large amounts of tissue for genotyping at the age of 1-10 days. This should be assessed in each case and should be made clear in the application for the animal experiment. Is there really a need for DNA at this age at all, or is the desire for tissue sampling based solely upon practical or economic considerations, or the claim of behavioural advantages by removing unwanted animals from a colony as quickly as possible?
10. Especially when invasive methods are used for sampling, any excess tissue should be stored so that the genotype can if necessary be characterised again without having to take a new sample. The size of the tissue sample should always be adjusted to the test method to be used, so no more tissue than necessary is removed.
11. Colonies of genetically modified animals are often large. It will always be tempting to choose methods (clinical studies, identification techniques, sampling procedures etc.) that are cheap and easy to perform.
12. There are a number of arguments for genotyping the animals in a genetically modified colony as early as possible:
    a. Unwanted animals can be humanely killed before weaning, reducing the number of cages, number of animals, risk of suffering caused by disease outbreaks and the occurrence of fighting/injuries, as well as the work burden on the employees
    b. The removal of some individuals from a litter will result in more milk being available, and will reduce stress, for the remaining young. In this way even the weakest can survive. These are often the genetically modified animals (e.g. homozygote knockouts), that have the greatest need for extra milk in this critical phase. Smaller litters result in larger and more robust young at weaning, and this is a significant argument in the improvement of breeding techniques for genetically modified and mutant animals.
    c. The dam is often pregnant at the time when toe clipping is to be performed, so there is less pressure on her if unwanted young are removed from the home cage (the psychological effects of the loss of these young must however also be taken into consideration). The tissue in the toes and tail tip of mice consists of cartilage, not bone, in the first two weeks of life, so toe and tail clipping at that age are probably comparable to the use of ear punching in older animals as regards av the experience of pain.
    d. Demands for effectivity should not be a main argument for choice of method, particularly if the method is considered to be a burden to the animals. Effective procedures are, however, often of little burden to animals, as they involve the minimum of restraint and therefore reduced stress. The effective management of an research animal unit is also about housing as few animals as possible, for as short a time as possible.
13. Opinions on the experience of pain in newborn animals (and humans) has coloured the debate on whether the more invasive methods are defensible or not. Experience pf pain (and therefore suffering) is conditional on two things: the nervous sytem must be

developed, so pain stimuli reach the brain cortex, and the animal must be conscious. Based upon measurement of the brain's electrical activity (EEG), Diesch *et al.* (2007) divide animal species into three different groups, depending upon how mature their nervous system is at birth. Marsupials (such as the kangaroo) are extremely immature at birth and conscious experience of pain is probably not developed until the animals are 120-180 days old. In other species (e.g. sheep), higher brain activity can be measured approximately 30 days before birth, but the lambs are maintained in a sleep-like state in the uterus by means of a range of substances produced in the brain. Behavioural studies show that these animals develop a full pain response gradually during the first week of life.

Rodents probably occupy a position between these extremes. Diesch *et al.* (2007) cite studies which Ellingsen & Rose (1970) conducted on the elektrical activity in the brain of young rats, and concluded that the animals did not show neurophysiological signs of the conscious experience of pain before 12-18 days after birth. From their own studies on young rats whose tails were clamped under anaesthesia, Diesch *et al.* concluded that conscious experience of pain does not normally occur earlier than 10-12 days after birth, with a gradual maturation process thereafter which lasts about one week.

One should all the same be cautious in exposing animals to painful stimuli during the phase when the nervous system is apparently still under development: studies on boys that were circumcised without anaesthesia revealed that they exhibited oversensitivity to the pain of vaccination up to 6 months later (Fitzgerald & Anand, 1993). In addition, there is evidence that the central nervous system is more (rather than less) sensistive to painful stimuli shortly after birth compared to later in life.

It is unclear how much analgesia is achieved by applying local anaesthesia during toe and tail clipping, or whether (and for how long) painkillers should be given after the procedure. There are practical problems in dosing these preparations to very young animals.

Studies of the brain's electrical activity in young rats cast doubts as to whether animals under 10 days of age are capable of the conscious experience of pain. This does not, however, rule out the possibility that these animals may feel pain later, even if anaesthesia has been used. Insufficient studies have been carried out to be able to assess whether the removal of the outermost joint of one toe affects the animal's normal way of life (e.g. climbing) or creates more than 'slight pain or discomfort of a highly temporary nature' (Norwegian Regulation on Animal Experimentation, §.2), e.g. in the form of the phantom pains that are described in humans following amputations, despite postoperative analgesic treatment. Neither is it possible to conclude whether the removal of a tail tip or a piece of the ear results in less pain than toe clipping. None of the methods are unproblematic. Tail clipping, on the other hand, differs from fra toe clipping in that it does not affect an organ that is in continual contact with the ground. The ears are too small in mice of the age in question to give enough tissue for quantitative genotyping.

7. Practice in other countries

There are few science-based reports from working groups that have evaluated the different methods of identifying and tissue sampling.

The *Joint Working Group on Refinement (JWGR, 2003)* in Great Britain recommended as a matter of principle that consideration should be taken to:

a. The source of the tissue
b. The size of the piece of tissue that is to be removed
c. The age of the animals
d. The need for local or general anaesthesia

The Group pointed out that the least invasive method should be used, and as little tissue as possible should be taken. Many of the least invasive methods are not used because there are traditions for using the more invasive ones, despite the existence of publications describing less invasive methods. Procedures should be reassessed regularly. The method used for genotyping should be discussed: Southern-blot hybridisations need more DNA than PCR techniques, but can be unavoidable if the number of transgenic copies must be identified. PCR should always be considered for routine genotyping of colonies, but it is important to avoid cross-contamination with other DNA.

In cases where both identification and genotyping are needed, the JWGR recommends the following (combined, if necessary, with an identification method):

	<2 weeks	3-4 weeks	>4 weeks
Saliva or faeces	√	√	√
Tail clipping	√/X	√	√/X
Ear punching	X	√	√
Blood	X	√	√
Toe clipping	X*	X	X

X: only in exceptional cases*

The JWGR was of the opinion that toe clipping probably incures pain and can reduce the animal's ability to grasp or groom. The Group concluded that it must not be used as a routine method of identification or tissue sampling for genotyping. In rare cases it may be unavoidable, e.g. where there are good scientific reasons for identifying mice of less than 14 days that are kept in isolators because of infection. In these cases, toe clipping may be the only practical method because of the animals' size and the need for biosecurity. Toe clipping should only be used as a last resort, and only one toe should be removed from one hindleg, under local anaesthesia. The tissue that is removed should be used for genotyping and the animals should not be subjected to further biopsy procedures. The method must not be used on animals older than 14 days, because other methods such as ear punching are then possible.

The JWGR mention several references in their guidelines on tail clipping, which Norecopa considers to be relevant when assessing toe clipping. The tail vertebrae begin to ossify at 2-3 weeks of age. There is evidence in the literature that there is bone substance even in the last millimetre of the tail. The skin and periosteum are rich in nervous tissue, and the JWGR concludes that tail clipping must be considered to be very painful, particularly when bony tissue is cut.

Norecopa's secretary has discussed the matter with the chair of the JWGR committe, Professor David Morton, UK. He recommends tail clipping (3-5 mm of tissue), which should normally be performed on mice before they are 2 weeks old and under full inhalational anaesthesia (e.g. isoflurane, N.B. not ether). Half of the sample DNA should be stored in case the tissue has to be retested, to avoid repeating the procedure (personal communication, cited with permission).

The ILAR Guide for the Care and Use of Laboratory Animals (NRC, 1996), which is used by, among others, the accreditation body AAALAC International (www.aaalac.org) states that toe clipping should only be used as an identification method in small rodents when no other method is possible in practice, and even then only be performed on 'altricial neonates'.

The Canadian organisation CCAC (Canadian Council on Animal Care, www.ccac.ca) have produced guidelines for the care of transgenic animals (1997) but these do not give specific recommendations on techniques for identification or tissue sampling.

An Internet search reveals that many local ethical committees describe the use of toe clipping. Som, such as the *Guidelines for biopsy procedures to facilitate identification and DNA-based molecular genotyping of rodents* from Emory University (2001) allow toe clipping on 8-12 day old animals without anaesthesia. In many cases, however, toe clipping is described as a method for identification alone, and is in such instances often cautioned against, with advice on using less invasive methods like tattooing or the use of transponders. This impression is supported by the replies that Norecopa has received to a posting on an international discussion forum for the laboratory animal community (CompMed) managed by the American laboratory animal science organisation AALAS.

8. Conclusions

1. Quantitative genotyping creates a need for relatively large amounts of DNA and therefore the use of invasive methods of tissue collection. This means in reality that amputations have to be performed on living animals. Amputations should therefore only be performed when tissue for quantitative genotyping is needed to characterise new genetically modified lines. Norecopa's Board believes that this principle should also apply to wild animals and fishk. Amputations should not be accepted as routine methods and special permission should be sought from the regulatory authorities to use them. Less invasive methods that need less DNA should therefore always be used for routine genotyping. It should be emphasised that animal protection organisations are in principle against amputations.

2. There are only two established methods that give sufficient tissue for quantitative genotyping of transgenic lines: tail clipping and toetip clipping. Although toetip clipping has the advantage that it also functions as an identification method, it is assumed to be more of a burden than tail clipping for the animal because it affects the locomotory apparatus which is more or less continually in contact with the ground. There are simple, non-invasive marking methods that can be combined with tail clipping, for example the application of a coloured dye to the skin in the armpits.

3. Norecopa's Board has registered that few institutions use toe clipping for genotyping, and that there is greater resistance to toe clipping than there is to tail clipping, even though both methods are controversial. The majority have replaced toe clipping as an identification method with other procedures, also for animals under 10 days of age, in agreement with the conclusions in the British JWGR report and the American ILAR Guide.

4. **Norecopa's Board is therefore of the opinion that toetip clipping, even with the refinements described in FDU's decision, should not be permitted. In those cases where it is absolutely necessary to undertake quantitative genotyping, tail clipping should be used (3-5 mm performed only once per animal) under anaesthesia, and with post-operative analgesia as long as this does not in itself create a greater burden for the animal. Further studies should be performed to identifiy the optimal anaesthesia and analgesia for the various methods of identification and tissue sampling. In cases where qualitative genotyping is adequate, less invasive methods should be employed, such as the collection of saliva, blood, faecal or hair samples, or (in larger animals) the material removed by ear punching.**

The minority of the Board has expressed their disagreement with this conclusion:

There is no evidence today that amputation of the tip of a toe is worse than amputation of the tip of the tail. Therefore toetip clipping should be permitted in situations where it is necessary to undertake genotyping at an early age. Early genotyping is necessary in cases where weak

offspring will have far better survival rates as soon as the litter size is reduced, in studies where genotyping is necessary before 14 days of age, or in cases where breeding is conducted in an isolator in connection with infection models. If the study design does not permit marking methods such as the use of pens, tattooing or other substances that will affect the study (e.g. toxicological, immunological or carcinogenic studies), toe clipping will function as a combined identification and biopsy method that together results in the least possible burden on the animal.

9. Literature

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6a

Van:
Verzonden: donderdag 16 november 2017 10:13
Aan:
Onderwerp: FW: ZonMw aanbiedingsbrief inventarisatie teenkootknip, oorknip
Bijlagen: ZonMw brief inventarisatie gebruik teenkootknip, oorknip.pdf; ZonMw Inventarisatie teenkootknip_in template_def.pdf; Bijlagen samengevoegd.pdf

Van: @zonmw.nl]
Verzonden: vrijdag 24 juni 2016 16:41
Aan:)
Onderwerp: ZonMw aanbiedingsbrief inventarisatie teenkootknip, oorknip

Geachte

Hierbij ontvangt u de ZonMw aanbiedingsbrief over de inventarisatie, van de drie aanvullende acties ten aanzien van de teenkootknip.

Bijgevoegd vindt u de volgende documenten:

- Aanbiedingsbrief ZonMw inventarisatie
- ZonMw inventarisatie
- Bijlagen bij de ZonMw inventarisatie

De stukken zijn vandaag per post verstuurd.

Met vriendelijke groet, namens

ZonMw
Laan van Nieuw Oost Indië 334
2593 CE Den Haag

Mail. l@zonmw.nl
Tel.

[redacted])

Van: [redacted]
Verzonden: donderdag 16 november 2017 10:16
Aan: [redacted]
Onderwerp: FW: kennismakingsgesprek

Van: [redacted]@zonmw.nl]
Verzonden: maandag 22 december 2014 15:49
Aan: [redacted]
Onderwerp: RE: kennismakingsgesprek

Hallo [redacted]

Ben iets later. Tot zo.

Groet,

[redacted]

Van: [redacted]@minez.nl]
Verzonden: maandag 22 december 2014 9:05
Aan: [redacted]
Onderwerp: RE: kennismakingsgesprek

Ha [redacted]

Is ook prima. Adres van het ministerie is Bezuidenhoutseweg 73. Ik zit op B-Noord, [redacted]
Ik zal je aanmelden.

Tot vanmiddag.

Met vriendelijke groet,
[redacted]

Directie Dierlijke Agroketens en Dierenwelzijn
[redacted]

Werkdagen:
Maandag, dinsdag en donderdag
(8.00-17.00 uur)
Op werkdagen bereikbaar onder:
[redacted]

Van: [redacted]@zonmw.nl]
Verzonden: maandag 22 december 2014 9:02
Aan: [redacted])
Onderwerp: RE: kennismakingsgesprek

Beste [redacted]

Dat is prima en vind het sympathiek dat je bij ZonMw wilt afspreken, maar wil met alle plezier naar EZ komen vanmiddag. Moet dan nog wel even weten waar ik precies moet zijn.

Groet,

Van: @minez.nl]
Verzonden: maandag 22 december 2014 8:58
Aan:
CC:
Onderwerp: FW: kennismakingsgesprek

Dag

Zullen we vandaag van 16.00-17.00 uur doen? Kom ik naar jou.

Met vriendelijke groet,

Sr. Beleidsmedewerker

Directie Dierlijke Agroketens en Dierenwelzijn

Werkdagen:
Maandag, dinsdag en donderdag
(8.00-17.00 uur)
Op werkdagen bereikbaar onder:

Van: @zonmw.nl]
Verzonden: donderdag 18 december 2014 19:39
Aan:
CC:
Onderwerp: FW: kennismakingsgesprek

Hoi

Ik ben officieel vanaf morgen vrij, daarom neem ik voor onderstaande suggesties even mee in cc, dat maakt een afspraak maken voor maandag of dinsdag wat eenvoudiger.

DPBII
We hebben voor.DPBII een vergelijkbaar rapport gemaakt als het overzicht DPB 2000-2011.
Alle projecten die onder DPBII zijn gehonoreerd zijn daarin opgenomen. Het rapport wil ik in januari aan OCW/DUO aanbieden als inhoudelijke eindverantwoording voor hun bijdrage aan DBPII. Ik wil het dan ook tegelijkertijd aan jullie aanbieden.

Fijne Feestdagen,

Van:
Verzonden: woensdag 17 december 2014 10:38
Aan: 'I
Onderwerp: RE: kennismakingsgesprek

Hoi

Ik hoor graag wat een eventuele deadline voor de teenknip en ook het symposium denk ik moet zijn, voor wat betreft de boekhouding.
Ik heb de vraag over ons totaal ZonMw budget nog uitstaan, maar ik hoop daar nog een reactie op te krijgen.
[redacted] werkt volgende week maandag en dinsdag ook nog en heeft verschillende mogelijkheden.

Een optie is van 10 tot 11 uur bijvoorbeeld of van 11 tot 12 uur op maandag 22 dec.
Eventueel eind van de middag van 16 tot 17 uur is mogelijk ook een optie.

Op dinsdag 23 dec. Zijn dezelfde tijdstippen ook een mogelijkheid.

Als je twee voorkeuren hebt of het maakt niet uit, dan stem ik dat af met [redacted] om het definitief te maken.

Gr [redacted]

Van: [redacted]@minez.nl]
Verzonden: dinsdag 16 december 2014 9:03
Aan: [redacted]
Onderwerp: RE: kennismakingsgesprek

Dag [redacted]

Ik werk t/m volgende week dinsdag (ivm de afspraak voor het kennismakingsgesprek) en dan weer vanaf 5 januari a.s.. Prima idee om zo'n gesprek te plannen.

M.b.t. de teenknip: hoe ziet het tijdpad er uit? Dit i.v.m. de noodzaak voor mij om hier intern in januari e.e.a. boekhoudkundig te regelen. Ik zal eens informeren
of er een bepaald tijdstip is waarop ik e.e.a. rond moet hebben.

Mooi dat je al gestart bent met de voorbereidingen van het symposium.

Tot slot: heb je al in jullie team of met [redacted] kunnen praten over een snellere behartiging van de innovatiemodule?

Met vriendelijke groet,
[redacted]
Sr. Beleidsmedewerker

Directie Dierlijke Agroketens en Dierenwelzijn
[redacted]

Werkdagen:
Maandag, dinsdag en donderdag
(8.00-17.00 uur)
Op werkdagen bereikbaar onder:
[redacted]

Van: [redacted]zonmw.nl]
Verzonden: maandag 15 december 2014 17:43
[redacted]
CC: MKMD
Onderwerp: kennismakingsgesprek

Beste [redacted]

We zijn bezig met de eerste kleine voorbereidingen voor het symposium begin tweede kwartaal. Daarnaast zijn we in gesprek met een paar mensen over alternatieven voor het teenknippen, maar daar heb ik nog wel wat tijd voor nodig. Daarnaast gaan we kennismakingsgesprekken inplannen met ondersteunende bureaus van NC en CCD. Even iets heel anders, het laatste overleg bij ons heb je al heel even kennis gemaakt met [redacted] de coördinator voor o.a. MKMD, maar we wilde nog een keer een kennismakingsgesprek in plannen.

Ik ga er vanuit dat de planning voor deze afspraak op januari uit zal komen gezien de naderende kersvakantie, maar als jij eventuele voorkeuren hebt dan hoor ik het graag.

Gr

Met vriendelijke groet,

Programmasecretaris

Telefoon:
Aanwezig op: ma, di, wo, do
E-mail: zonmw.nl

ZonMw
Laan van Nieuw Oost Indië 334, 2593 CE Den Haag
Postbus 93245, 2509 AE Den Haag
www.zonmw.nl

Van:
Verzonden: donderdag 16 november 2017 10:19
Aan:
Onderwerp: FW:

Van:
Verzonden: dinsdag 13 januari 2015 15:24
Aan:
Onderwerp: RE:

Ja, volgens mij ook.

Met vriendelijke groet,

Sr. Beleidsmedewerker

Directie Dierlijke Agroketens en Dierenwelzijn

Werkdagen:
Maandag, dinsdag en donderdag
(8.00-17.00 uur)
Op werkdagen bereikbaar onder:

Van:
Verzonden: dinsdag 13 januari 2015 15:14
Aan:)
Onderwerp: RE:

Ministerie van EZ
Directie Agrokennis
Cluster Onderzoek en Kennisvalorisatie
Bezuidenhoutseweg 73, 2594 AC Den Haag
telefoon
e-mail: @minez.nl

Aanwezig op maandag t/m donderdag

Van:)
Verzonden: dinsdag 13 januari 2015 15:13
Aan:
Onderwerp: RE:

Met vriendelijke groet,

Sr. Beleidsmedewerker

Directie Dierlijke Agroketens en Dierenwelzijn

Werkdagen:
Maandag, dinsdag en donderdag
(8.00-17.00 uur)
Op werkdagen bereikbaar onder:

Van:
Verzonden: dinsdag 13 januari 2015 14:46
Aan:
Onderwerp: RE:

Hallo

Bedoel je dat het toch lukt om het in de brief mee te nemen? M.a.w. is wat aanlevert daar voldoende voor?

Ministerie van EZ
Directie Agrokennis
Cluster Onderzoek en Kennisvalorisatie
Bezuidenhoutseweg 73, 2594 AC Den Haag
telefoon
e-mail: @minez.nl

Aanwezig op maandag t/m donderdag

Van:
Verzonden: dinsdag 13 januari 2015 13:56
Aan:
Onderwerp: RE: eventueel later?

Ik krijg morgen van een mail waarin een aanvulling op het programmavoorstel wordt gegeven. Die voeg ik bij laatstgenoemd voorstel en dan kan de brief etc. worden opgesteld en de afhandeling in gang gezet worden.

Met vriendelijke groet,

Sr. Beleidsmedewerker

Directie Dierlijke Agroketens en Dierenwelzijn

Werkdagen:
Maandag, dinsdag en donderdag
(8.00-17.00 uur)
Op werkdagen bereikbaar onder:

Van:)
Verzonden: donderdag 8 januari 2015 11:53
Aan:
Onderwerp: RE: eventueel later?

Hallo
Het liefst natuurlijk in een brief. Ook vanwege die voorjaarsnota binnenkort.

Ministerie van EZ
Directie Agrokennis
Cluster Onderzoek en Kennisvalorisatie
Bezuidenhoutseweg 73, 2594 AC Den Haag
telefoon
e-mail: @minez.nl

Aanwezig op maandag t/m donderdag

Van:
Verzonden: donderdag 8 januari 2015 11:40
Aan:
Onderwerp: eventueel later?

Eind 2014 werd duidelijk dat ZonMw ook een call uit moet gaan zetten om tot de ontwikkeling van een alternatief voor de teenknip bij zeer jonge muizen te komen.

Met vriendelijke groet,
Sr. Beleidsmedewerker

Directie Dierlijke Agroketens en Dierenwelzijn

Werkdagen:
Maandag, dinsdag en donderdag
(8.00-17.00 uur)
Op werkdagen bereikbaar onder:

9

Van:
Verzonden: donderdag 16 november 2017 10:16
Aan:
Onderwerp: FW: bedrag teenknip

Van:
Verzonden: donderdag 8 januari 2015 11:51
Aan:
Onderwerp: bedrag teenknip

,

Ik moet deze maand intern de budgetoverheveling van onze directie naar de onderzoeksdirectie regelen voor het programma Meer Kennis met Minder Dieren (budget 2e programmaperiode). Weet je al wat er nodig is voor de aanvullende activiteiten? Ik doel op het bedrag dat nodig is voor de workshop niet-humane primaten en ik doel op de call die uitgezet moet worden voor de ontwikkeling van een alternatief voor de teenknip.

Graag z.s.m. je reactie, zodat ik e.e.a. in werking kan zetten intern. Dank je wel. En... de allerbeste wensen voor 2015!!

Met vriendelijke groet,
s
Sr. Beleidsmedewerker

Directie Dierlijke Agroketens en Dierenwelzijn

Werkdagen:
Maandag, dinsdag en donderdag
(8.00-17.00 uur)
Op werkdagen bereikbaar onder:

Van:
Verzonden: donderdag 16 november 2017 10:17
Aan:
Onderwerp:

Urgentie: Hoog

Van:
Verzonden: dinsdag 13 januari 2015 10:33
Aan:
Urgentie: Hoog

Graga je reactie. Dank.

Met vriendelijke groet,

Sr. Beleidsmedewerker

Directie Dierlijke Agroketens en Dierenwelzijn

Werkdagen:
Maandag, dinsdag en donderdag
(8.00-17.00 uur)
Op werkdagen bereikbaar onder:

11

Van:
Verzonden: donderdag 16 november 2017 10:19
Aan:)
Onderwerp: FW: Reactie ZonMw op aanvullende vragen MKMD

Urgentie: Hoog

Mail over tenenknip

Van: zonmw.nl]
Verzonden: woensdag 14 januari 2015 8:44
Aan:
CC:
Onderwerp: Reactie ZonMw op aanvullende vragen MKMD
Urgentie: Hoog

Beste

- ...
- ....

2. Alternatieven voor teenknip uit het Plan van Aanpak dierproeven en alternatieven van 28 februari.

We hebben enkele wetenschappelijke artikelen op dit terrein bekeken (zie bijlage) en met enkele experts gesproken over mogelijke alternatieven voor deze specifieke vraag (nadere gespreken met experts op dit terrein ook over de grens zijn afgesproken, maar nog niet gerealiseerd). Alternatieven zijn zover we nu hebben kunnen achterhalen niet direct voor handen, maar er is meer tijd nodig dit verder uit te werken en te onderzoeken. Uit de literatuur en gesprekken komt wel naar voren dat er geen consensus (of bekendheid, ook Europees) is over welke methoden voor genotypering en identificatie er allemaal voor handen zijn en welke methoden je in bepaalde situaties het beste kunt gebruiken.

Welke methoden gebruikt worden binnen instituten binnen Nederland wisselt afhankelijk van o.a. de onderzoeksvraag en lijkt niet overal hetzelfde.

Tegelijkertijd zou deze inventarisatie gebruikt kunnen worden om te kijken welke ideeën/richtingen kansrijk zouden zijn voor ontwikkeling van alternatieven voor de teenknip.

Kosten € 110.000

Gr

Met vriendelijke groet,

Programmasecretaris

Aanwezig op: ma, di, wo, do
@zonmw.nl

ZonMw
Laan van Nieuw Oost Indië 334, 2593 CE Den Haag
Postbus 93245, 2509 AE Den Haag
www.zonmw.nl

12

Van:
Verzonden: donderdag 16 november 2017 10:23
Aan:
Onderwerp: FW: E-mail verzenden: Aanvraagformulier DG Agro overboeking DAD naar DAK ivm MKMD 2015-2017.doc
Bijlagen: Aanvraagformulier DG Agro overboeking DAD naar DAK ivm MKMD 2015-2017.doc

-----Oorspronkelijk bericht-----
Van:
Verzonden: dinsdag 20 januari 2015 12:15
Aan:
Onderwerp: E-mail verzenden: Aanvraagformulier DG Agro overboeking DAD naar DAK ivm MKMD 2015-2017.doc

Je kan i.v.m. argumentatie desgewenst nog opnemen helemaal aan het begin:

In het Plan van aanpak Dierproeven en Alternatieven d.d. 28 februari 2014

Ik stuur je dadelijk ook nog een scan van de relevante pagina's van het plan waar dit staat.

Gr.

Hierbij de tekst van het overboekingsformulier. Daaruit kan je dan onder 'toelichting' de korte stuk tekst halen die je nodig hebt.
Als je nog meer nodig hebt hoor ik het graag.

Gr.

Uw bericht kan nu met het volgende bijlagen of koppelingen worden verzonden:

Aanvraagformulier DG Agro overboeking DAD naar DAK ivm MKMD 2015-2017.doc

Aanvraagformulier aangaan van financiële verplichting DG Agro

Algemeen	
Naam (beleids)medewerker	
Directie/Cluster	DAD/DW
Projectnaam voorstel	ZonMw onderzoeksprogramma 'Meer Kennis met Minder Dieren'

Toelichting voor DG/Directeur/MT over beleidsrelevantie (en keuze opdrachtnemer bij opdracht):

€ 110.000,-.

Dit formulier betreft alleen de interne overboeking

Projectnummer in bestedingenplan	101488
Operationele doelstelling	U16.30
Instrumentnummer	250260
Instrumentnaam	Dierproeven
Financiën	
Opdracht[1] of Subsidie[2]	Opdracht ☐ N.v.t. Subsidie ☐ N.v.t.

[1] Aanbestedingsprocedures bij opdrachten: boven € 25.000 minimaal 3 offertes en boven € 125.000 excl. BTW Europese aanbesteding

[2] Bij subsidies denk aan checliststaatsteuntoets (of Verklaring beperkte steun), wettelijk kader en aantal keren incidentele subsidie (maximaal 4 keer)

Aanvraagformulier aangaan van financiële verplichting DG Agro			
Argumentatie indien Opdracht niet volgens de aanbestedingsprocedure[1]	N.v.t.		
Argumentatie indien voor Subsidie geen staatsteunmelding[2] nodig	N.v.t.		
Risico inschatting subsidie-ontvanger	Op basis van op relevante informatie gemaakte risicoanalyse, schat ik het risico van misbruik en oneigenlijk gebruik in op: Laag ☐ Gemiddeld ☐ Hoog ☐ N.v.t.		
Begroot bedrag in bestedingenplan	€ (op jaarbasis)		
Bedrag van de verplichting, incl.BTW	€ (totaal voor 3 jaar)		
Kasritme van de (voorschot)betalingen	2012: €	2013: €	2014: €
	2015: €	2016: € -	2017: € -
Startdatum project	**Begin januari 2015**		
Einddatum project	**Eind december 2017**		
Beoordeling aanvraag			
Advies Staatsteuncoördinator (bij subsidies)	N.v.t.		
Advies BC Agro Controller Directie			

[1] Aanbestedingsprocedures bij opdrachten: boven € 25.000 minimaal 3 offertes en boven € 125.000 excl. BTW Europese aanbesteding

[2] Bij subsidies denk aan checliststaatsteuntoets (of Verklaring beperkte steun), wettelijk kader en aantal keren incidentele subsidie (maximaal 4 keer)

[redacted]

Van: [redacted]
Verzonden: donderdag 16 november 2017 10:24
Aan: [redacted]
Onderwerp: FW: Uw brief is verzonden: 15009273 Opdrachtbrief ZonMw Meer kennis minder dieren vervolgprogramma
Bijlagen: DOMUS-15009273-Opdrachtbrief ZonMw Meer kennis minder dieren vervolgprogramma.PDF; Bijlage 1 bij opdrachtbrief ZonMw MKMD 2015-2017 RODA standpunt.docx; Bijlage 2 bij opdrachtbrief ZonMw 053-B RODA Verslag ethische reflectie teenknip (2).docx

-----Oorspronkelijk bericht--
Van: [redacted]
Verzonden: dinsdag 3 februari 2015 10:43
Aan: [redacted]

[redacted]@zonmw.nl'
Onderwerp: FW: Uw brief is verzonden: 15009273 Opdrachtbrief ZonMw Meer kennis minder dieren vervolgprogramma

Beste [redacted]

Deze brief is verzonden.

Groeten,
[redacted]

Ministerie van EZ
Directie Agrokennis
Cluster Onderzoek en Kennisvalorisatie
Bezuidenhoutseweg 73, 2594 AC Den Haag
telefoon 0[redacted]
e-mail: [redacted]@minez.nl

Aanwezig op maandag t/m donderdag

13a

Ministerie van Economische Zaken

> Retouradres Postbus 20401 2500 EK Den Haag

Het bestuur van ZonMw
Postbus 93245
2509 AE Den Haag

Directoraat-generaal Agro
Directie Agrokennis

Bezoekadres
Bezuidenhoutseweg 73
2594 AC Den Haag

Postadres
Postbus 20401
2500 EK Den Haag

Factuuradres
Postbus 16180
2500 BD Den Haag

Overheidsidentificatienr
00000001003214369000

T 070 379 8911 (algemeen)
www.rijksoverheid.nl/ez

Behandeld door
[redacted]
[redacted]minez.nl

Ons kenmerk
DGA-AK / 15009273

Uw kenmerk
8 december 2014
Programmavoorstel Meer kennis minder dieren

Bijlage(n)
2

Datum 30 JAN. 2015
Betreft Opdrachtbrief ZonMw Meer kennis minder dieren vervolgprogramma 2015 t/m 2017

Geacht bestuur,

Bij schrijven d.d. 8 december 2014 heeft u mij uw programmavoorstel 'Meer Kennis met Minder Dieren 2015 tot en met 2017' aangeboden. Mijn hartelijke dank hiervoor!

I[redacted]

Ik verzoek u voor de teenknip drie acties uit te werken:

- U wordt verzocht een zorgvuldige schatting te maken van de omvang van de toepassing van de teenknip;
- U wordt verzocht de activiteiten uit te voeren zoals beschreven in de mail van 14-01-2015 (w.o. consensus protocol en implementatieplan);
- Tot slot ontvang ik graag van u nog een aanvullend voorstel en begroting voor een separate (aanvullende) module waarin u een call uitzet om een alternatieve methode voor de teenknip te ontwikkelen.

Directoraat-generaal Agro
Directie Agrokennis

Ons kenmerk
DGA-AK / 15009273

[redacted]

In verband met voornoemde acties rond de teenknip doe ik u bijgaand het standpunt van het Regulier Overleg Dierproeven en Alternatieven over de teenknip toekomen. U treft eveneens aan het verslag van de ethische reflectie die op dit gebied in 2014 heeft plaatsgevonden.

[redacted]

- [redacted];
- en tot slot in aanvulling hierop een bedrag van maximaal € 110.000,- voor de in de mail d.d. 14-01-2015 beschreven activiteiten op het gebied van de teenknip. Ik ga ervan uit dat de zorgvuldige schatting van de omvang van de toepassing van de teenknip binnen het laatste bedrag meegenomen wordt. Een resterend deelbudget wordt in onderling overleg tussen u en de contactpersoon van EZ herbestemd.
- Ik verzoek u mij voor de nieuwe module voor de ontwikkeling van een alternatief voor de teenknip nog een aanvullend voorstel te doen toekomen. Hiervoor krijgt u te zijner tijd in een separate brief dan nog aanvullend budget.

[redacted]

2015: € [redacted]
2016: € [redacted]-
2017: € [redacted]-

Directoraat-generaal Agro
Directie Agrokennis

Ons kenmerk
DGA-AK / 15009273

Voor de uitvoering van dit programma zijn de werkafspraken Planning en control afspraken VWS, NWO en ZonMw van toepassing. De financiering voor dit programma loopt via het Ministerie van VWS.

Ik verzoek u tijdens en over de uitvoering van het programmavoorstel nauw contact te onderhouden met de contactpersoon van de directie Dierlijke Agroketens en Dierenwelzijn van EZ,

Ik wens u veel succes toe met de uitvoering van dit programma,

De Staatssecretaris van Economische Zaken
namens deze:

ir. M.A.A.M. Berkelmans,
wnd. directeur Agrokennis

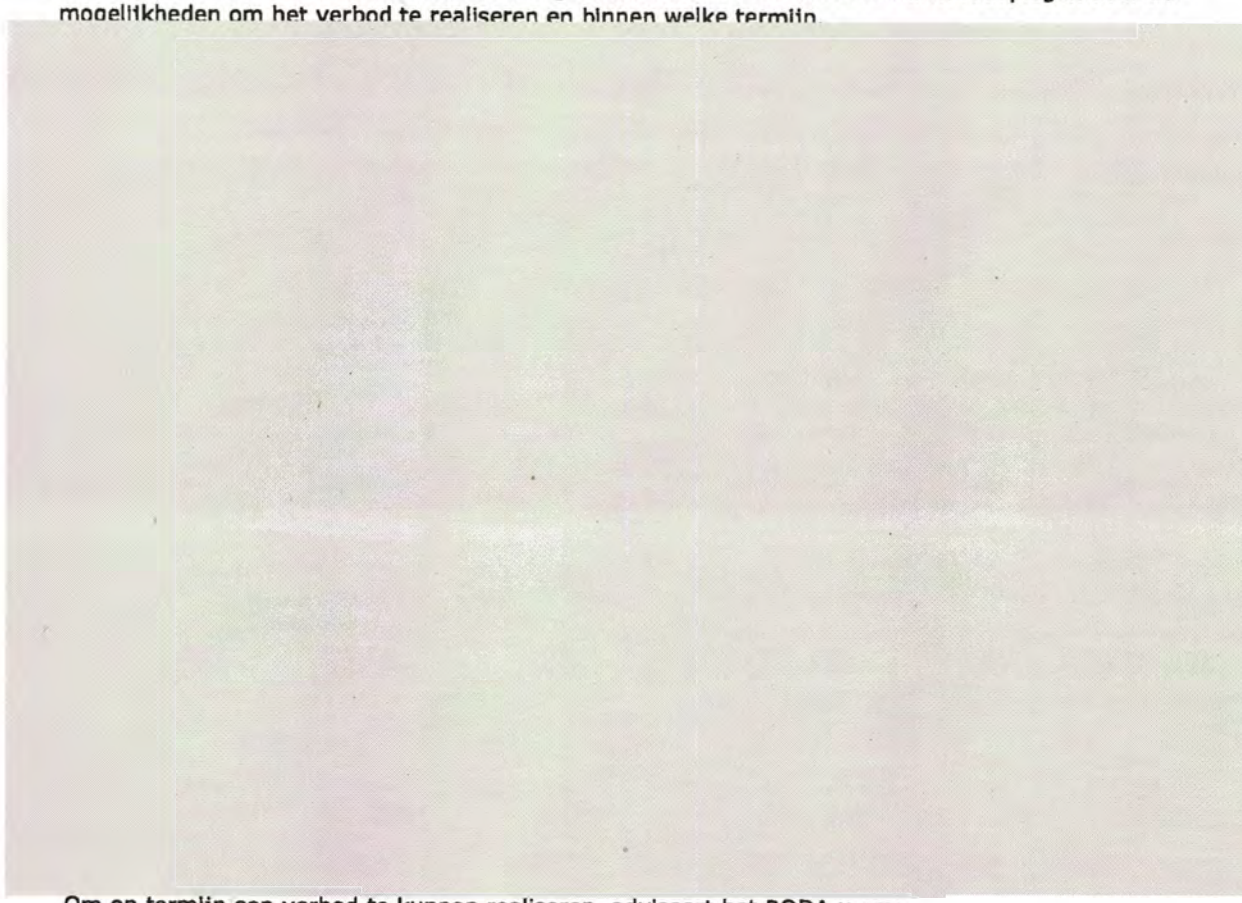
13c

Aan de Staatssecretaris
van Economische Zaken
Mw. S.A.M. Dijksma
Postbus 20401
2500 EK DEN HAAG

Den Haag, 20 november 2014

Geachte mevrouw Dijksma,

In het Plan van aanpak Dierproeven en alternatieven (TK 2013-2014, 32 336, nr. 27) heeft u aangekondigd dat u voornemens bent de teenknip bij muizen te verbieden. Hierbij hebt u gesteld dat voor een dergelijk verbod eerst een aantal voorwaarden vervuld zal moeten zijn, zoals het hebben van goede alternatieve identificatiemethoden. U gaf aan in 2014 het RODA te willen raadplegen over de mogelijkheden om het verbod te realiseren en binnen welke termijn



Om op termijn een verbod te kunnen realiseren, adviseert het RODA u om:

1. via een enquête de omvang van de toepassing van de teenknip in beeld te brengen
2.

3. [redacted]

4. [redacted]

Het RODA is van mening u hiermee goede vervolgstappen te hebben aangereikt, die op termijn invulling kunnen geven aan uw wens om de teenknip te verbieden.

Met vriendelijke groet,

[redacted]

Mevr. Drs. Dineke (E.J.) Mulock Houwer

Van:
Verzonden: donderdag 16 november 2017 10:25
Aan:
Onderwerp: FW: enkele vragen rond de teenknip

Van: zonmw.nl]
Verzonden: donderdag 12 februari 2015 10:53
Aan:)
CC:
Onderwerp: RE: enkele vragen rond de teenknip

Hoi,

Ik vroeg mij al af, maar heb al wel wat informatie naar toegestuurd ☺
Twee artikelen die zo wie zo erg informatief zijn voor dit terrein.
Gr

Van: @minez.nl]
Verzonden: donderdag 12 februari 2015 10:39
Aan:
CC:)
Onderwerp: enkele vragen rond de teenknip

Je kunt de beantwoording van deze vragen richting ons nu laten zitten, daar ZonMw inmiddels verzocht is om de acties rond de teenknip uit te voeren. Was een mail van voor ons verzoek aan ZonMw. Dat zag ik net pas.

Gr.

Beste

Een collega van mij, , is bezig met de teenknip.

Het RODA heeft geadviseerd om allereerst de toepassing van de teenknip in beeld te brengen en de staatssecretaris heeft in het AO van 3 december jl. toegezegd voortgang op dit gebied te zullen rapporteren voor de zomer.
s hiermee bezig. In dit verband vraagt zich af of jullie (al) weten waar deze ingreep wordt toegepast en wie zij zou kunnen benaderen om aan de benodigde informatie (frequentie van toepassing, onder welke voorwaarden, alternatieven etc.) te komen.

Heeft ZonMw inmiddels al de aantallen en andere informatie in beeld? Dat zou ons namelijk erg helpen.

Ik cc. Wil je je antwoord a.u.b. aan haar sturen?

Dank je!

Groet,

15

Van:
Verzonden: donderdag 16 november 2017 10:25
Aan:
Onderwerp: FW: E-mail verzenden: Verslag AO 3 december 2014 kst-32336-40.pdf
Bijlagen: Verslag AO 3 december 2014 kst-32336-40.pdf

-----Oorspronkelijk bericht-----
Van:
Verzonden: maandag 23 februari 2015 14:43
Aan: '

Onderwerp: E-mail verzenden: Verslag AO 3 december 2014 kst-32336-40.pdf

Bij deze het vastgestelde verslag van het AO van eind 2014. I.v.m. de niet-humane primaten en wat uitspraken over ZonMw Je leest het wel.

Gr.

Uw bericht kan nu met het volgende bijlagen of koppelingen worden verzonden:

Verslag AO 3 december 2014 kst-32336-40.pdf

Van:
Verzonden: donderdag 16 november 2017 10:28
Aan:
Onderwerp: FW: artikelen teenknip en alternatieven
Bijlagen: dahlborn_k_Verbost_Felasa artikel teenknip.pdf; Bonaparte_D_FELASA_genotyping rodents.pdf

Urgentie: Hoog

.nl]
Verzonden: dinsdag 31 maart 2015 18:25
Aan:
Onderwerp: artikelen teenknip en alternatieven
Urgentie: Hoog

Hoi

Deze twee artikelen bedoel ik. De eerste is met inbreng van

Met vriendelijke groet,

Programmasecretaris

Telefoon:
Aanwezig op: ma, di, wo, do
E-mail: @zonmw.nl

ZonMw
Laan van Nieuw Oost Indië 334, 2593 CE Den Haag
Postbus 93245, 2509 AE Den Haag
www.zonmw.nl

Working Party Report

Laboratory Animals
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FELASA guidelines for the refinement of methods for genotyping genetically-modified rodents

A report of the Federation of European Laboratory Animal Science Associations Working Group

Dolores Bonaparte (Convenor)[1,2], Paolo Cinelli[3,4], Eleni Douni[5,6,7], Yann Hérault[8,9,10], Alex Maas[11,12], Pirjo Pakarinen[13,14], Matti Poutanen[13,14], Mirentxu Santos Lafuente[15,16] and Ferdinando Scavizzi[17,18]

Abstract

The use of genetically-modified (GM) animals as research models continues to grow. The completion of the mouse genome sequence, together with the high-throughput international effort to introduce mutations across the mouse genome in the embryonic stem (ES) cells (www.knockoutmouse.org) facilitates an efficient way to obtain mutated mouse strains as research models. The increasing number of available mutated mouse strains and their combinations, together with the increasing complexity in the targeting approaches used, reinforces the need for guidelines that will provide information about the mouse strains and the robust and reliable methods used for their genotyping. This information, however, should be obtained with a method causing minimal discomfort to the experimental animals. We have, therefore, compiled the present document which summarizes the currently available methods for obtaining genotype information. It provides updated guidelines concerning animal identification, DNA sampling and genotyping, and the information to be kept and distributed for any mutated rodent strain.

Keywords

genotyping, husbandry, polymerase chain reaction, refinement, sampling

[1]SPCAL, Sociedade Portuguesa de Ciências em Animais de Laboratório
[2]Instituto de Medicina Molecular, Lisbon, Portugal
[3]SGV, Schweizerische Gesellschaft für Versuchstierkunde
[4]Institute of Laboratory Animal Science, University of Zürich, Zurich, Switzerland
[5]HSBLAS, Hellenic Society of Biomedical and Laboratory Animal Science
[6]Institute of Immunology, Biomedical Science Research Center 'Alexander Fleming', Athens, Greece
[7]Department of Agricultural Biotechnology, Agricultural University of Athens, Athens, Greece
[8]AFSTAL, Association Française des Sciences et Techniques de l'Animal de Laboratoire
[9]Institut Clinique de la Souris (ICS), INSERM U964, CNRS UMR7104, Université de Strasbourg, Illkirch, France
[10]Institut de Genétique de Biologie Moléculaire et Cellulaire (IGBMC), INSERM U964, CNRS UMR7104, Université de Strasbourg, Illkirch, France
[11]NVP, Nederlandse Vereniging voor Proefdierkunde
[12]Department of Cell Biology, Erasmus Medical Center, PO Box 2040, 3000 CA Rotterdam, The Netherlands
[13]ScandLAS, Scandinavian Society for Laboratory Animal Science
[14]Institute of Biomedicine and Turku Center for Disease Modeling, University of Turku, Turku, Finland
[15]SECAL, Sociedad Española para las Ciencias del Animal de Laboratorio
[16]Centro de Investigaciones Energéticas Medioambientales y Tecnológicas (CIEMAT), Madrid, Spain
[17]AISAL Associazione Italiana per le Scienze degli Animali da Laboratorio
[18]Consiglio Nazionale delle Ricerche, Istituto di Biologia Cellulare, European Mouse Mutant Archive, Monterotondo, Italy

Corresponding author:
D Bonaparte, Instituto de Medicina Molecular Animal Facility, Av. Prof. Egas Moniz, Edif. Egas Moniz, 1649-028 Lisboa, Portugal.
Email: doloresbf@fm.ul.pt

As a starting point, we have decided to collect information on the genotyping practices applied in various European countries, in order to identify common problems and pitfalls faced and to better direct our recommendations. For this purpose, we have conducted a survey focused on genotyping-related procedures used. We have collected 158 responses from 25 countries (see Supplementary information http://lan.sagepub.com/content/suppl/2013/05/28/0023677212473918.DC1/LAN473918_Supplementaryd_data.pdf), which will be acknowledged in this report when appropriate. Only three replies were received with regard to rats, which seems to show that this species is still infrequently used for genetically-modified (GM) models. From the responses to this survey, we could conclude that the procedures are not harmonized among countries or even among different institutes within one country, and that the common practice is not always in accordance with the latest scientific findings.

Collection of samples for genotyping

Classical genotyping procedures for rodents rely on polymerase chain reaction (PCR) and Southern blot analyses of the DNA specimens obtained from tissue samples of individual animals. This approach is also supported by our survey, as shown in the Supplementary information (please see http://lan.sagepub.com/content/suppl/2013/05/28/0023677212473918.DC1/LAN473918_Supplementaryd_data.pdf).

Traditionally, the most widely used methods for obtaining the tissue material for genotyping are tail, ear, or toe biopsies. Because collecting such biopsies involves a given amount of discomfort for the animals, alternative methods for DNA collection have been proposed in order to reduce it. However, according to the survey of the present study (Table 1) these 'alternative' methods are not widely used, possibly due to problems related to accuracy, reproducibility, and practicability as identified in our survey and on comparative tests carried out by the members of this Working Group in their facilities.

Guidelines for the refinement and reduction of procedures involving GM rodents, also including recommendations for DNA sampling, have been published before by other Working Groups.[1,2] One of the aims of the present task, thus, was to re-evaluate those guidelines in the light of new scientific evidence and to provide up-to-date information about the most adequate DNA sampling methods. These updated guidelines are general and will apply to the majority of strains. The recommended techniques should, however, be adjusted in case of specific phenotypic constraints that should render them impossible, inadequate, or more severe.

Identifying the animals to genotype

Animals have to be individually identified so that the determined genotypes can be assigned to the correct individuals. The FELASA Working Group on Rodent Identification has recently issued recommendations on this subject, which should be considered for detailed information. Coincidently, some reliable identification techniques provide a tissue sample as by-product that can be used to extract DNA. Our primary recommendation is, therefore, that whenever there is the need to both identify an individual and collect a DNA sample for genotyping, a method should be chosen that meets both goals. This will avoid the use of two invasive techniques and will minimize handling, which is shown to be a major source of discomfort in this process.[3,4] The survey performed by our group (see Table 1) showed that this rationale is not always followed by researchers and, thus, intensive education is needed in this regard.

Collecting DNA samples for genotyping

Overall, we recommend using the least invasive method that provides an adequate DNA sample in terms of quality and quantity to perform a robust genotyping procedure as described in the previous section. Whenever possible, this method should, at the same time, provide highly reliable identification.

Table 1. Methods used for sampling/genotyping and for identification both in newborns up to P17, and from P18 onwards, presented as percentage of survey responders.

	Sampling/genotyping		Identification	
Method	P0–P17	P18–adult	P0–P17	P18–adult
Tail biopsy	42	79	NA	NA
Ear notch/punch	5	43	8	64
Phalanx removal	12	4	20	6
Blood	<1	13	NA	NA
Hair	0	<1	NA	NA
Faecal pellets	0	0	NA	NA
Oral swab	0	0	NA	NA
Ear tag	NA	NA	4	33
Tattoo/colour marking	NA	NA	15	6
Transponder	NA	NA	0	6
Isolation in a single cage	NA	NA	0	<1
UV light	NA	NA	<1	0
No response	43	6	53	8

Total number of responders was 158. UV: ultraviolet, NA: not applicable.

In addition to the invasiveness of the process in itself, the associated handling and restraining, which have been shown to be a major stress factor, should be considered. Moreover, species, strain, age of the animal and its phenotype must also be acknowledged. One should also make every effort to base judgement on scientific data, and interpret concepts such as invasiveness, pain and discomfort from the perspective of the animal, avoiding excessive anthropomorphization.[5,6]

Those sampling techniques that are also identification methods are thoroughly described in the Report of the Working Group on Rodent Identification. In these cases, our report will focus mainly on the aspects related to the collection of samples for genotyping.

Ear biopsy. Ear biopsy is a widely used method where a piece of tissue is removed from the mouse pinna. The method provides both a DNA sample and a means of identification, based on the location of the biopsy. The method seems not to induce major signs of discomfort[3] and, because the ear is not very vascularized, bleeding is also minimal if present at all. Surprisingly, only 43% of the scientists in our survey reported using ear biopsy as a DNA sampling method on animals older than 17 days (Table 1). Moreover, 64% used this method for identification purposes upon genotyping, indicating that around 20% of the scientists perform two invasive procedures where they could use just one.

There is a vast array of instruments that can be used for the procedure, ranging from small scissors to sophisticated ear punches with attached microtubes for easy tissue collection. The 2 mm version of the punchers is recommended for yielding enough DNA for PCR, while decreasing the risk of losing the identification mark by healing.

Before the ear is erect and when the pinna is still too small, this method is not possible or not accurate for identification purposes. Although varying with strain and individuals, the ear is usually sufficiently developed for this technique at around 14 days of age. After this threshold, ear biopsy should be the method of choice whenever there is a need for both permanent identification and collection of DNA for genotyping.

Tail biopsy. Tail biopsy consists of the amputation of a small portion of the distal tail. Despite being reliable and the most traditional tissue biopsy method for obtaining DNA for genotyping (79% of the users in our survey use the method in mice over 17 days of age), this method does not provide any identification, thus requiring a concurrent procedure for this purpose. For this reason alone, it should be discouraged as first option when permanent identification is required unless an adequate DNA sample cannot be obtained by a more recommended technique.

If, however, this method is to be chosen, the following points should be considered:

1. Recent studies have shown that taking the biopsy on days 14 to 17 results in a less ossified sample which can be significantly shorter than what was recommended in the previous guidelines;[7,8]
2. At this age, a 3 mm long biopsy will provide enough DNA for PCR. However, Southern blot analysis might require longer (5 mm) biopsies;[3,8,9]
3. Most mice react to tail snip and this reaction increases in intensity and number with age, probably due to vertebral maturation;[7,10]
4. Some guidelines[2,8] and institutions recommend the use of analgesia or anaesthesia when a tail biopsy is taken at the age of 21 days or older. However, results of a recent study indicate that isoflurane anaesthesia did not reduce acute behavioural responses to tail biopsy in BALB/c and C57BL/6 mice.[10] Furthermore, it remains controversial whether local analgesia is effective and whether general analgesia or anaesthesia are advantageous in terms of global welfare as these procedures are not without adverse physiological impact.[2,9,10]

Because of the above, we recommend that tail biopsy should be used only when the techniques that account for both sampling and identification do not provide a tissue sample that is adequate for the genotyping protocol (e.g. Southern blot), or when the identification aspect of such techniques is redundant or not valid (e.g. the animal is already identified or requires a particular identification method other than ear marking, such as a transponder).

In cases where tail biopsy is used:

1. Mice should preferably be between 14 and 17 days old;
2. Sample should be no longer than 3 mm, except when the genotyping technique is known to require a large amount of DNA;
3. A very sharp instrument should be used, with a clean, precise cutting gesture;
4. In case of bleeding, haemostasis should be enforced;
5. In older animals, the benefits of analgesia or anaesthesia should be evaluated.

Distal phalanx biopsy. Distal phalanx biopsy consists of the removal of the distal phalanx of a newborn animal, that is then used as a source of DNA. This method also provides a means of identification, and is described in detail in the literature[11–13] and in the report of the FELASA Identification Working Group.

A similar, non-refined method generally known as 'toe clipping' has been largely used in the past, regardless of the extent of the biopsy or the age of the animal. Owing to its mutilating aspect, that method was gradually set aside and is now discouraged or even prohibited in several European countries and institutions. In our survey, 17 of the 72 scientists reported Animal Ethics Committee restrictions regarding toe clipping at their sites of research, other than age-related.

Recent studies[3,12–14] have shown that the refined distal phalanx biopsy of newborns, if properly performed, does not seem to affect mice more than tail or ear biopsy, neither in the short nor the long term.[12,13] Conversely, tail biopsy has been shown to affect mice, at least in the short term.[10,15]

Although the technique is possible earlier, postnatal day (PND) 7 seems to be the preferred age for distal phalanx biopsy. At this age, toes are already well separated and easy to access,[4,12,13] the ossification process is not yet completed,[11] and the animals have reduced movements, which facilitates the accurate biopsy. There are national and institutional guidelines pointing out other ages as the limit, such as PND 12.[16] However, we were unable to find scientific grounds for those.

With many studies requiring the genotyping of newborns and early genotyping becoming a growing trend, it is important to re-evaluate the sampling options available. At very early ages, ear sampling is not possible and tail biopsy requires concurrent marking techniques. In the light of current knowledge, distal phalanx biopsy (refined version) is, therefore, the method of choice to genotype newborns because it provides both identification and a source of good quality DNA on a sole intervention with minimum disturbance of the animals.

Accordingly, we recommend this method with the following safeguards:

1. At the time of the biopsy, the animals should be approximately seven days old;
2. Only the most distal phalanx of only one toe per paw should be removed;
3. No further biopsies should be performed.

We would like to take this opportunity to advocate early, pre-weaning genotyping. This approach has many advantages: the animals are easier to handle; some tissue samples yield more DNA as they are less ossified; and genotyping results are available before the weaning date, allowing for the better planning of experiments and management of the colonies. As an extra benefit, housing costs decrease as surplus animals can be managed before weaning time.

Blood. Blood can be used to extract DNA for genotyping, although it may present a few technical problems.[17] It may also be a convenient method for genotype determination by using flow cytometry or microscopy-based techniques.[18] In effect, 13% of the researchers who responded to our survey had used blood to determine the genotype (Table 1). However, blood sampling does not enable mouse identification. Hence, it can only be considered as a refinement in terms of sampling for genotyping in animals already identified, identified with non-invasive methods, or requiring an identification method that does not provide a DNA sample (e.g. transponders).

When this method is chosen, the amount of blood collected should respect the recommended volumes, and the collection technique should be the least invasive that is still appropriate for the required volume and genotyping method.[19]

Hair follicles. Hair follicles plucked from the animal can be used as a source of DNA for genotype identification. This technique would reduce the discomfort associated with the more invasive sampling techniques.[3,20] Unfortunately, its throughput is low and there is a high risk of cross-contamination between samples of different animals.[3,20] Moreover, it does not provide a means of identifying the animals.

Owing to the above constraints, the technique is not appropriate for routine genotyping of large animal colonies. However, it can be considered for the following cases:

1. Low numbers of animals already identified, requiring an identification method that is not a sampling method (e.g. transponder) or that can be identified through a non-invasive method;
2. Older animals already identified, requiring an identification method that is not a sampling method (e.g. transponder) or that can be identified through a non-invasive method;
3. Re-sampling for confirmatory testing;
4. Colonies for which an invasive method can pose a threat (e.g. bleeding disorders).

Based on our survey, less than 1% of the scientists use hair follicle DNA for mouse identification (Table 1).

Colonic and rectal cells. Colonic and rectal cells, collected either by means of a rectal swab or scrape[3,21] or from faecal pellets[22–24] can be used as a DNA source for genotyping. While we would not think of rectal swabs or scrapes as non-invasive, the collection of faecal pellets could be a method to consider. Owing to possible DNA degradation, faecal pellets are to be obtained freshly,[24] or at the latest within 24 h.[22] The faeces need to be individually collected and more than one faecal pellet per animal is normally required.

For the above reasons, this method is not practical when sampling large numbers of animals. In addition, like hair plucking, the method does not provide a means for identification. Stool sampling may however be considered in the same situations described for hair sampling. None of those responding to our query reported using this method for genotyping.

Cells from the oral mucosa. To obtain oral cells for DNA sampling, the oral cavity may be scraped[25] or swabbed.[26] Alternatively, the oral cavity can be flushed with a small amount of sterile water, although only a small amount of cells is retrieved by this technique from weanling mice.[27] This method is widely used for human DNA collection, as it is virtually non-invasive in humans. However, there are reports of mice biting their tongues during the process, and the samples are often tinted by blood.[3] Thus, the size and design of the collection tools should be carefully considered to keep the method non-invasive when applied in small rodents. Although this method could be considered useful for large colonies due to higher throughput and lower risk of contaminations when compared with the previous two methods, the expected low amount of DNA obtained is a limitation. Furthermore, the method also does not provide a means of identifying the animals. For this reason, we suggest that it should also be reserved for the situations listed for hair follicles and rectal cells.

None of the survey inquirers reported using this method.

Summary of sampling recommendations

In conclusion, when considering sampling for genotyping, we recommend the following:

- Work quietly and be gentle in order to minimize the stress associated with handling and restraint.
- Whenever possible, use a methodology that simultaneously identifies the animals and yields tissue for genotyping.
- Work carefully to avoid the need for repeating the procedures: identify the animals in a reliable way; collect the samples cautiously, and avoid cross-contamination.
- Whenever possible, opt for early genotyping.
- Collect the minimum amount of tissue that can still produce enough DNA for the selected genotyping method.
- Store and ship samples properly, to avoid DNA degradation. Consider extracting immediately and storing and/or shipping purified DNA instead of tissue.

Taking into consideration the age of the animals and the need to permanently identify them or not, Table 2 summarizes our recommended sampling methods for the most common situations. However, if the genotyping method requires a higher amount of DNA, or the recommended technique is inadequate due to physiological constraints of the model or scientific aspects of the project, another sampling method may have to be considered.

Development of an optimized genotyping protocol

An optimized genotyping protocol is essential to ensure fast and reliable mouse identification. PCR and Southern blot-based methods are currently by far the most commonly used methods. This was confirmed by our survey, with 92% of the respondents reporting the use of PCR followed by 10% using quantitative PCR, 9% Southern blot/dot blot and 4% single nucleotide polymorphism (SNP) detection as routine methods (see Supplementary information http://lan.sagepub.com/content/suppl/2013/05/28/0023677212473918.DC1/LAN473918_Supplementaryd_data.pdf).

Table 2. Recommended methods for the collection of samples for genotyping by polymerase chain reaction.

	Sampling of animals requiring permanent ID	Batch sampling of animals not needing permanent ID*	Re-sampling, small numbers of animals not needing permanent ID*
Day 5–Day 7 (Day 7 is preferred)	Distal phalanx biopsy	Distal phalanx biopsy	Not applicable
Day 8–Day 13†	Distal phalanx biopsy	Distal phalanx biopsy (best) Tail biopsy (≤3 mm)	Not applicable
Day 14–Day 17	Ear biopsy	Ear biopsy (best) Tail biopsy (≤3 mm)	Hair/faeces/saliva Ear biopsy‡
After Day 17	Ear biopsy	Ear biopsy	Hair/faeces/saliva Ear biopsy‡

*Also animals that, for experimental or institutional reasons, require an identification (ID) method that does not provide a sample (e.g. transponders).
†No information from the literature for this age interval – use good judgement.
‡Except for animals with previous ear marks that could be compromised by that ear biopsy.

The advantages of the PCR-based methods are the speed and the low amount of DNA needed, which translate into a low amount of tissue required for sampling. Only 1–2 ng of DNA are needed for PCR, while Southern blot analysis requires microgram quantities of DNA. We provide below a few considerations for successful PCR and Southern-based genotyping of GM rodents.

DNA purification

Proper DNA purification is a critical step for setting up a PCR-based method for mouse identification, especially when a new genotyping protocol is established. After establishment of the method, the use of highly pure genomic DNA is not always necessary. This is also highlighted in our survey where only 18% of the respondents reported to the use of commercial kits for DNA purification as a routine (see Supplementary information http://lan.sagepub.com/content/suppl/2013/05/28/0023677212473918.DC1/LAN473918_Supplementaryd_data.pdf).

Currently, there are several well-established methods available for extracting DNA from tissue biopsies. According to the experience of this Working Group and the results of our survey (see Supplementary information), the two most commonly used methods for tissue digestion are the use of proteinase K in the presence or absence of sodium dodecyl sulphate (SDS), and the use of alkaline lysis (HotSHOT method[28]). In addition to these methods, there are several commercial DNA extraction kits available which tend to be easy to work with but are expensive to use. The survey confirmed that more than 75% of the respondents use proteinase K digestion for routine DNA isolation. We suggest three protocols which should deliver high-quality genomic DNA from tissue biopsies for PCR genotyping (see Supplementary information):

As crude tissue extract is typically used, DNA quality is not routinely analysed while genotyping GM mouse colonies. Supplementary Table S4 summarizes the most important DNA isolation protocols, the most common tissue sample and the respective procedure(s) for genotyping, i.e. PCR or Southern blot methods. Independent of the approach used for tissue collection, it is important to prevent contamination between the samples by means of proper working practices (using clean punching devices and forceps, avoiding contamination by hair, etc.) and to store samples properly to prevent DNA degradation. Where possible, it is also advisable to store part of the DNA to enable a repeat genotyping to be performed without the need for additional sampling. This is particularly important if the test is not optimized, or if problems are anticipated.

Polymerase chain reaction

This is a technique to amplify even a single copy of a particular DNA sequence, generating millions of copies. It is by far the most widely used method for genotyping routinely, a trend reflected in the results of the survey: 92% of the total number of responders reported using this method.

General considerations. For transgene detection, 150–800 base pair long PCR products are typically used for efficient amplification. In multiplex PCR methods it is important to amplify fragments with similar lengths, with a preferred length difference of 50–100 bp, in order not to favour amplification of the shorter product. It is essential to set up a robust mutation-independent (control) PCR method for a single-copy gene expected to be present in any mouse strain. This is used as an internal control for successful DNA extraction, and to detect putative contaminants that interfere with DNA amplification. With the control genes, a product is added that should always be present, thus preventing false-negative results (Figure 1a and b). Examples of such genes and primer pairs which have been successfully used are provided in Table 3.

For the genotyping of mutant mice produced with homologous recombination (such as knock-out and knock-in mice), typically three primers are used in PCR (see Figure 1c). In this case, the primers have to be designed so that the first two (forward and reverse) amplify a gene-specific region and the third primer (reverse) is specific for the selection cassette. In the normal situation, gene-specific primers are not able to amplify a product in the targeted locus (fragment is too long) and therefore amplify only the wild-type allele. Also, in this PCR strategy, products should have a difference in length of about 50–100 bp.

In the maintenance of large animal colonies, it is important that the PCR conditions are optimized in such a way that several genotyping PCRs can be performed simultaneously with the same programme. A detailed protocol for successful genotyping as well as a troubleshooting guide for PCR are provided as Supplementary document A (please see http://lan.sagepub.com/content/suppl/2013/05/28/0023677212473918.DC1/LAN473918_Supplementaryd_data.pdf).

Southern blot analysis

Large amounts of DNA (7–15 μg) of high purity, suitable for restriction enzyme cleavage, are needed for each digest. The resulting DNA fragments are separated by electrophoresis, transferred to membranes, and the transgenic product is visualized after hybridization with a specific labelled probe. The technique takes

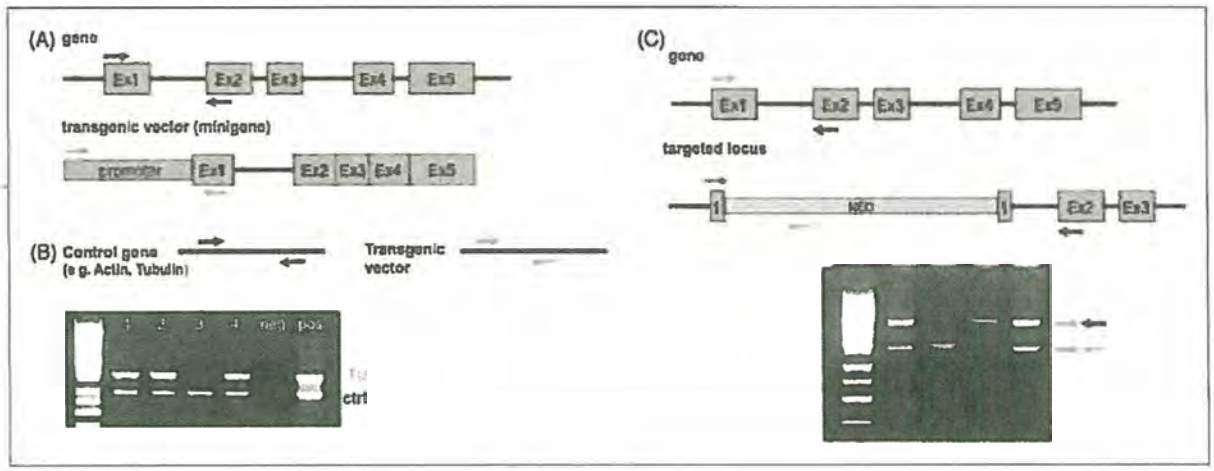

Figure 1. Use of control genes in polymerase chain reaction.

Table 3. Selection of genes and primers that can be used as internal controls.

IL-6; interleukin-6 gene		
IL-6	For	5'-TTC-CAT-CCA-GTT-GCC-TTC-TTG-G-3'
IL-6	Rev	5'-TTC-TCA-TTT-CCA-CGA-TTT-CCC-AG-3'
PCR product		170 bp
Immunoglobulin heavy chain-joining region		
Igh-j	For	5'-TGT-CCA-GGG-TCT-ATC-GGA-CT-3'
Igh-j	Rev	5'-GTT-TTT-CCT-CTG-TAC-CCG-AC-3'
PCR product		290 bp
B1 receptor gene; bradykinin receptor, beta 1		
B1	For	5'-CTC-AGG-GAG-GCC-AGG-ATG-TG-3'
B1	Rev	5'-TCA-GCG-GGG-TCA-TCA-AGG-CC-3'
PCR product		340 bp
VAX 1; ventral anterior homeobox gene		
VAX 1	For	5'-CGT-AAT-CAA-TTG-CAA-CAG-CGA-G-3'
VAX 1	Rev	5'-AGA-AGG-AGG-GTG-GGA-AAA-GAA-G-3'
PCR product		400 bp
Grem 1; Gremlin 1 gene		
Grem 1	For	5'-ATG-AAT-CGC-ACC-GCA-TAC-ACT-G-3'
Grem 1	Rev	5'-TCC-AAG-TCG-ATG-GAT-ATG-CAA-CG-3'
PCR product		500 bp
Rb1; Retinoblastoma gene		
Rb1	For	5'-GGC-GTG-TGC-CAT-CAA-TG-3'
Rb1	Rev	5'-AAC-TCA-AGG-GAG-ACC-TG-3'
PCR product		650 bp

at least three days to complete and can be performed using non-radioactive labelling.

Southern blot requires more DNA and more tissue, but it is a required method in some instances. In the case that transgenics are generated by pronuclear injection of DNA, as it is the case for many lines carrying the Cre-expressing transgene, Southern blot is a powerful tool for determining the properties of transgene integration. It can be used: (1) to discriminate between lines when different integration sites occur in each line; (2) to determine the number of sites of integration; (3) to determine the transgenic copy number; (4) to verify the integrity of the transgene and the orientation of the tandem repeats; and (5) to detect homozygous individuals. Southern blot can also give information of the locus structure at the integration site and the overall integrity of the recombined transgene.

Thus, Southern blot is a useful technique to be used for the characterization of founders of a new transgenic line. When mutants are generated by gene targeting strategies, Southern blot is the gold standard for the identification of homologous recombination events in embryonic stem (ES) cells. In this case it is conceivable that, after obtaining germline competent chimeric animals upon injection of targeted ES cells into blastocysts, the mutations in the F1 generation are first tested by PCR, and upon expansion of the colony, only a representative number of animals is tested by Southern blotting, avoiding unnecessary collection of large tail biopsies.

Other genotyping methods

Quantitative realtime PCR. Realtime PCR allows the accumulation of the amplified product to be detected and measured as the reaction progresses. For the detection of PCR products, a fluorescent molecule is included in the reaction. This fluorescent molecule (DNA-binding dyes as SYBR Green or fluorescently labelled sequence-specific primers or probes as TaqMan) reports an increase in the amount of DNA with a proportional increase in the fluorescent signal, measuring the amount of amplified product in realtime.

In realtime PCR, the amount of amplification product is determined after each cycle, enabling a much more accurate quantification. It has been used for

genotyping,[29–31] for copy number detection of a transgene,[32] and for determination of zygosity.[33] However, for the latter purpose it should be used with caution as the method is not very reliable with high copy number of the transgene of interest. Realtime PCR also provides an accurate and sensitive method to determine the ultimate level of the Cre-specific gene disruption in conditional mutants with the Cre-loxP system.[34]

Genome-wide genotyping. Genome-wide genotyping enables scanning of the whole genome using polymorphic genetic markers for the identification of genetic differences between inbred or GM mice. Because genome-wide genotyping requires high-quality genomic DNA and adequate DNA quantity, genomic DNA is usually prepared from tail biopsies using a proteinase K/SDS lysis buffer method followed by a phenol–chloroform extraction and isopropanol precipitation. Subsequently, the genetic polymorphisms of test and control samples are amplified by PCR and ran on agarose gels or genetic analysers. The whole genome is scanned with high-density polymorphic markers, such as simple sequence length polymorphisms (SSLPs) and SNPs. We recommend using at least 2–3 genotyping markers, about 30 cM in between each other, to assess the identity of the chromosomes.

SSLPs, also known as microsatellite markers, are serial di-, tri-, tetra- or penta-nucleotide repeats, where the number of repeat units varies among the different inbred strains. They are easily amplified by PCR using primers derived from unique sequences flanking the repeat units, and the length variance (>4 bp) can be detected on 4% agarose gels. Usually the PCR product size of SSLP markers ranges from 80 to 250 bp. A dense genetic map of the mouse with numerous SSLPs that distinguish different inbred strains has been established[35–37] which is freely accessible through various websites (Supplementary Table S5; please see http://lan.sagepub.com/content/suppl/2013/05/28/0023677212473918.DC1/LAN473918_Supplementaryd_data.pdf) so that every molecular biology laboratory can easily establish its customized panel of SSLPs. Even though SSLP genotyping is easy and cost-effective, genome-wide genotyping has been further upgraded by the use of SNPs.[38]

SNPs are single base pair mutations that occur at a specific site in the DNA sequence and are the most common type of genetic variation among inbred strains. In addition, SNP is the genotyping method of choice for screening point mutations induced with the alkylating agent *N*-ethyl-*N*-nitrosourea (ENU).[39,40] A plethora of SNP genotyping platforms is currently available.[41] High-throughput technologies genotype thousands of SNPs simultaneously, including Affymetrix GeneChip arrays,[42] and Illumina BeadArray technology.[43] Both platforms use the same basic principle of hybridization of genomic DNA fragments to a fixed probe. These microarray technologies require 250–750 ng of genomic DNA per sample and only one sample is simultaneously tested for all markers per assay. Such methods offer a dense scan of the mouse genome with multiplex SNP genotyping and have mainly application in genetic mapping screens but are not considered cost-effective for genotyping a large panel of mice. Other customized methods for SNP genotyping, such as Pyrosequencing,[44,45] Applied Biosystems TaqMan approach,[46] and Sequenom MALDI-TOF mass spec[47] are readily applied to a large number of samples, with relatively low running costs. Since SNP genotyping requires expensive equipments, reagents, experienced personnel, and significant analysis capabilities, it is usually offered as a service by institutional core facilities (Supplementary Table S5).

Applications of the genome-wide genotyping methods

Until recently, genome-wide genotyping was exclusively applied on genetic mapping for the identification of the causal mutation that was responsible for the observed phenotype. During the last decade, a forward genetics or phenotype-driven approach (phenotype to gene) was established in various research centres worldwide through genome-wide random ENU mutagenesis. This approach involves screening of thousands of mutagenized mice for mutant phenotypes and subsequent identification of the mutated gene. With the advances in genome sequencing and the discovery of thousands of polymorphisms in the mouse genome, the process of genetic mapping is now much more efficient and significantly less time-consuming than a decade ago.

Genome-wide genotyping has also important applications in the evaluation of the genetic purity and contamination of any mouse genetic background.[38] Mutant lines maintained on an inbred background should be monitored regularly for evidence of genetic contamination due to accidental mismating. A genetic quality monitoring of breeders with approximately 50 genome-wide polymorphic markers offers detection of massive genetic contamination. However, subtle changes of the genetic background could be detected only by a dense scan of the genome with SNPs spreading all over the genome that are available from different providers (i.e. Affymetrix [www.affmetrix.com], Illumina [www.illumina.com]). Ultimately the next generation sequencing allows a complete overview of the mouse genome of interest and to compare with the original inbred genetic background (http://www.sanger.ac.uk/resources/mouse/genomes/) A successful genetic quality control monitoring system is essential for the maintenance of well-established strain characteristics

and the reproducibility of experimental data between different groups over time, taking advantages of the whole genome genotyping techniques described previously.

Moreover, genome-wide genotyping accelerates dramatically the development of congenic strains which are mutant lines repeatedly backcrossed to an inbred strain. In this genome-based strategy, named speed congenics, offspring are genotyped with a genome-wide panel of genetic markers, and animals with optimal genotype (those that have retained the mutation and the largest proportion of recipient background) are selected for the next breeding step. The optimal breeding strategy appears to be a 10 cM genome scan of 20–50 males per backcross generation, which requires approximately 150 markers per mouse. The advantage of this approach is the fact that it minimizes the number of backcrosses required to establish a quality congenic mouse strain as early as with the fifth backcross generation[48,49] instead of the 10 generations that are needed if a traditional breeding scheme is followed. Speed congenics provides a fast, reliable and cost-effective way for backcrossing as it reduces by half the time that is required for the backcrossing of mutant lines, i.e. from 3 to 1.5 years and minimizes the animals that are needed for backcrossing.

Relevant information for mutated rodent strains

Most mutants have been generated on a hybrid genetic background, and it is well known how important the contribution of the genetic background is to the phenotype of a given mutation. Thus, it is essential to keep adequate records of detailed information on the type of mutation, methods for genotype identification, genetic background, etc. for all GM mouse strains generated, and to pass this information together with the mouse strain to any user and collaborator. An example of such a data sheet is included as a Supplementary document B (please see http://lan.sagepub.com/content/suppl/2013/05/28/0023677212473918.DC1/LAN473918_Supplementaryd_data.pdf).

Strain identification and type of mutation

Every mutant strain must have a name providing precise information on the affected gene, the type of mutation, the parental inbred strain(s) and the code for the laboratory where this mutant originates from, which can be requested from the Institute for Laboratory Animal Research (ILAR) (Supplementary Table S5). Nomenclature rules are set by the International Committee on Standardized Genetic Nomenclature for Mice and the Rat Genome and Nomenclature Committee.[1] This nomenclature needs to be used in order to provide unquestionable information about the type of mutation introduced (Supplementary Table S5).

For any transgene, a schematic presentation describing in detail the transgene structure used is essential, including precise information about the gene regulatory region and the protein coding regions used. For transgenic mice, the insertion site is not typically analysed. However, if known, the flanking sequences of the integration site should be given.

In case of a gene trap, the genomic locus of the insertion should be provided together with the description of the targeted locus. This information is available from the ES cell providers, such as the International Gene Trap Consortium (Supplementary Table S5). If available, it is also essential to provide the information about the insertion site of the gene trap.

For a targeted insertion, a detailed description by one nucleotide resolution of the targeted locus, including positions of exons, introns, possible sites for loxP- and Frt-sequences, probes and restriction enzyme sites used for confirming the targeted allele by Southern blotting, and PCR primers used for routine genotyping of the targeted locus should be provided, and preferably indicated also in a schematic drawing.

In general, it is preferable to attach the same information electronically in order to facilitate the analysis of the modified locus. If available, the original publication where the mouse model was described for the first time should also be included. Emphasis should also be given to report the site and type of a putative antibiotic selection gene and/or the reporter gene inserted into the locus. As an example, a schematic presentation of a hypothetical, multipurpose allele is presented in Figure 2.

Identification of the genotype

For efficient colony maintenance, and to promote scientific collaboration concerning the various mouse strains, protocols developed for the genotyping of rodents should be simple, easy and as robust as possible. Moreover, these demands should be met by a method causing minimal stress to the animals.

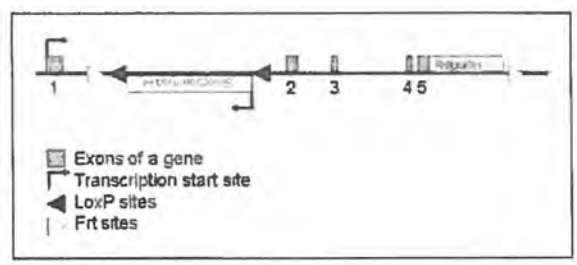

Figure 2. Example of a multipurpose gene knock-in.

Once selected, the most appropriate genotyping protocol should be recorded along with the strain information, so that it could be reproduced without problems.

As mentioned previously, routine genotyping of mutated mouse strains are preferably performed by PCR. PCR would allow the distinction of wild-type, heterozygous and homozygous animals. However, in transgenic rodents this is not typically possible, due to the unknown integration site. In those cases, two independent primer pairs are designed to amplify a region with similar size: one specific for the transgene and another unrelated pair of primers are designed to demonstrate the proper amount and quality of the DNA in the specimen. This information should be adequately recorded and provided.

Equally important is the use of a reliable animal identification system, as no genotype information is valid without an unquestionable way to link it to an individual at any time and location within no matter how large animal facilities. It is, therefore, of the utmost importance that, not only are the mice well identified, but also the identification code and correspondent genotype are recorded and conveyed when transferring the animals to a different location.

Sometimes it is possible to identify the mutants by the phenotype. However, discriminating the mutant and wild-type littermates by the macroscopic appearance only is not always advisable. This is due to the fact that the phenotypic features might vary between the individuals, especially between generations and in the different genetic backgrounds.

Genetic background

The original genetic background (donor strain) onto which the mutation was originally introduced – e.g. embryo donors for the transgenic mouse production and the ES cell line used for homologous recombination or for the production of the gene trap clone – should be described. Since phenotypic changes in the mutant rodent also depend on the genetic background, it is essential to provide a summary of the breeding history of the mouse model. International laboratory codes should be kept, as well as the terms Fxx, to indicate the number (xx) of intercrosses, or Nyy to indicate the number (yy) of backcross generations from a donor to a new recipient background.[1] As importantly, the gender of the carrier should be indicated as defined by the International Committee on Standardized Genetic Nomenclature.

Typically, backcrossing a mutation generated in a donor background for up to 10 generations into a new recipient background is considered necessary to produce a homogenous inbred mouse strain. However, it should be remembered that the genomic regions close to the genotyped mutation do not typically undergo homologous recombination; those regions are, thus, not backcrossed, containing alleles of the donor background for genes located at the vicinity of the mutation. If additional techniques are used, such as speed congenics,[48] a description of the genetic markers used and located proximal to the mutation should be provided. Alternatively, performing a survey of the genetic background using high-density genetic markers such as SNP arrays developed by commercial companies would be recommended.[42,43]

The user needs to remember that some strains have particular characteristics (e.g. they are more prone to certain types of diseases). Thus, when using backcrossed mutants it is often needed to monitor unexpected influences of the genetic background. These possible changes should also be recorded along with the strain information.

Husbandry characteristics

To obtain and provide a short summary of the known phenotypic changes affecting the health and general fitness and therefore, putatively required to apply for project approval by animal ethical committees and to adequately maintain the strains – is of the utmost relevance. Information on fertility of both genders, and any special considerations concerning the breeding strategies to be applied, should be monitored and conveyed. Similarly, information on the viability and fertility of both heterozygous and homozygous mutants should be recorded and provided for appropriate breeding strategy, housing, handling and maintenance of the mouse line.

Data on the history of the microbiological status of the rodent strain are of key importance for a smooth colony transfer between laboratories, and to recognize any health problems that might not be linked to the mutation itself. Indeed, even the bacterial gut flora could interfere with the mutation introduced (in particular for genes controlling metabolism or immunity) and impact the health or the response of the mutant.

Concluding remarks

The increasing number and complexity of GM rodent strains has determined a corresponding increase in the number and complexity of the procedures required for their successful generation, maintenance and use. It has also, fortuitously, resulted in increasing scientific studies aimed at refining these procedures in terms of effectiveness and of animal welfare. Unfortunately, many scientists are still making use of 'traditional' genotyping-associated procedures, even if these are not the

most appropriate in terms of animal welfare or even of success.

With the present guidelines, we aim to further implement the concept of the 3Rs on genotyping-associated procedures, refining and harmonizing them in the light of the latest scientific findings, and according to the current scenario of animal research.

We believe that the proposed recommendations will lead to better animal welfare while improving scientific results and saving time and resources.

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