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International survey on the use and welfare of zebrafish *Danio rerio* in research

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A survey was conducted regarding zebrafish *Danio rerio* use for scientific research with a focus on: anaesthesia and euthanasia; housing and husbandry; breeding and production; refinement opportunities. A total of 98 survey responses were received from laboratories in 22 countries in Europe, North America, South America, Asia and Australia. There appears a clear and urgent need to identify the most humane methods of anaesthesia and euthanasia. Aversive responses to MS-222 were widely observed raising concerns about the use of this anaesthetic for *D. rerio*. The use of anaesthesia in fin clipping for genetic identification is widely practised and there appears to be an opportunity to further develop less invasive methods and refine this process. Optimization (and potentially standardization) of feeding is an area for further investigation. Given that diet and body condition can have such profound effects on results of experiments, differences in practice could have significant scientific implications. Further research into transition between dark and light phases in the laboratory appears to represent an opportunity to establish best practice. Plants and gravel were not considered practical by many laboratories. The true value and benefits need to be established and communicated. Overproduction is a concern both from ethical and financial viewpoints. There is an opportunity to further reduce wastage of *D. rerio*. There are clear concerns and opportunities for the scientific community to work together to further improve the welfare of these important laboratory models.

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Key words: 3Rs; anaesthesia; breeding; environmental enrichment; euthanasia; refinement.

INTRODUCTION

It has been estimated that >3250 institutes spread through 100 countries work with zebrafish *Danio rerio* (Hamilton 1822) (Kinth *et al.*, 2013). Globally, it remains unclear just how many *D. rerio* are used annually (maybe >5 million, see below). Furthermore, it seems that this use is set to increase, particularly with the advent of rapid clustered regularly interspaced short palindromic repeats (CRISPR)–*cas9* gene (<http://www.yourgenome.org/facts/what-is-crispr-cas9/>) editing that will affect many *D. rerio* models and probably increase overall research intensity (Lawrence, 2016).

Best welfare practice is clearly a critical criterion for optimizing the use of these animals. There have been many efforts into establishing good practice for husbandry and care (Lawrence, 2007; Westerfield, 2007; Reed & Jennings, 2011). Just how well these

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guidelines are applied in practice, however, remains unclear, with significant anecdotal evidence that globally many laboratories operate their own standards and regional differences in legislation drive very different approaches to many important aspects (*e.g.* euthanasia methods). This diversity provides an opportunity for the *D. rerio* research community to share these practices and provide a baseline for progressing with a policy of continuous improvement in animal welfare (Prescott & Buchanan-Smith, 2007; Lawrence *et al.*, 2016; Lidster *et al.*, 2016). The fundamental principles of replacement, reduction and refinement of laboratory animal use (3Rs) are now widely accepted and there is increasing focus on the importance of refinement to improve welfare and deliver the highest quality science. In order to progress the 3Rs, it is important to understand the extent of current practices in the global community.

To establish this baseline of current conditions and practices, a short survey was conducted across a subset of *D. rerio* laboratories from 22 countries (see Appendix SI, Supporting Information) and a range of size of facility from <500 broodfish to >10 000. The aim of the survey was to gather information on the use of *D. rerio* in research, including their anaesthesia and euthanasia, housing and husbandry and breeding and production, with an overall aim to better understand common practices and identify opportunities for refinement.

MATERIALS AND METHODS

A qualitative survey was conducted amongst laboratories using *D. rerio* in research (Appendix SI, Supporting Information). Survey invitations were emailed to 485 laboratories between the period of 19 February and 31 March 2016, identified from the Zebrafish Information Network (ZFIN; www.zfin.org). A call on LinkedIn (Zebrafish group; www.linkedin.com) elicited a further 17 responses. A total of 98 survey responses were fully completed from 22 different countries [Fig. 1(a)]. Survey respondents included scientists, principle investigators, technicians and facility managers. Survey responses were received from workers in 14 of the top 25 most popular research subjects [Fig. 1(b)] in which *D. rerio* are the key research model (Kintz *et al.*, 2013). Further, approximately equal numbers of responses were received from laboratories ranked by size of standing broodstock (<500, <1000, <5000, <10 000 and >10 000 *D. rerio*). Together, this range suggests the 98 survey responses were likely to be broadly reflective of the current global *D. rerio* research community and probably represent the more progressive laboratories.

The survey was split into four key themes relating to the welfare of *D. rerio*: anaesthesia and euthanasia; housing and husbandry; breeding and production; refinement opportunities. The survey was constructed and administered using SurveyMonkey (www.surveymonkey.org). The data acquired were managed according to a standard data management plan for NC3Rs office-led data sharing projects and anonymized to protect the identity of individuals and laboratories. The survey was multi-layered and the job responsibility of the respondent steered their route through the survey and the questions asked. Responses were collated for each question and are reported as number of responses. The number of responses to each question is, therefore, unique and reported in absolute terms. No statistical analysis or manipulation was performed and the anonymized data are reported (responses that could identify individual laboratories have been redacted from Appendix SI, Supporting Information). Each response represents a single laboratory as most respondents self-identified in the survey.

RESULTS

ANAESTHESIA AND EUTHANASIA

The survey showed that anaesthesia was used in most laboratories (89 of 98 respondents) for a range of scientific procedures, with 76 (of 89) respondents reporting

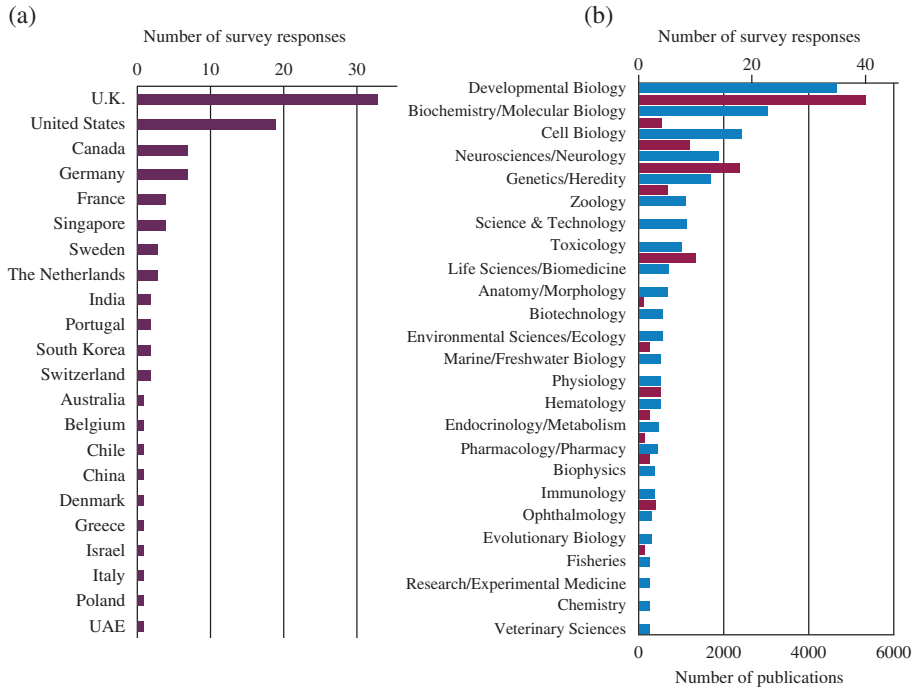


FIG. 1. (a) Geographical frequency distribution of *Danio rerio* survey respondents. (b) Research areas represented in the survey showing the number of publications in *D. rerio* research (■; reported in Kirth *et al.*, 2013) representative of the number of survey responses (■).

the use of anaesthesia in fin clipping for genetic identification and 71 (of 88) for the first step of euthanasia. Other procedures involving anaesthesia (45 of 89) included (but not limited to) imaging, electrophysiology, intra-peritoneal injections, cell transplantation, tissue collection and biometric measurements. The majority (83 of 89) of survey respondents routinely use ethyl 3-aminobenzoate methanesulphate (MS-222, or tricaine) as an anaesthetic agent (Fig. 2). The survey cannot distinguish which regions these represent. In 77 (of 88) laboratories where MS-222 is used, the solution is routinely buffered. It should be noted that MS-222 is the only agent approved for fishes in some regions.

To understand the rationale behind the choice of anaesthetic agent, survey respondents were asked what factors they take into consideration when choosing an anaesthetic using a weighted response (Fig. 3). The most important factors were the ease of use and efficacy of the anaesthetic agent; the least important factor was the cost.

The depth of anaesthesia is monitored by response to stimuli (76 of 89), visual assessment (74 of 89), ventilation rates (59 of 89) and posture (42 of 89). Over half of respondents (61 of 98) reported observing some adverse effects when using anaesthesia such as rapid swimming, escape response, gasping, avoidance and colour change. Due to the questionnaire structure, it is not possible to attribute this observation to any particular anaesthetic agent, or life stage, but it seems likely that most refer to MS-222. MS-222 was also the most commonly used anaesthetic agent for euthanasia in larvae (<5 days old; 52 of 89) and broodstock (67 of 88). Ice was also used as a method of euthanasia

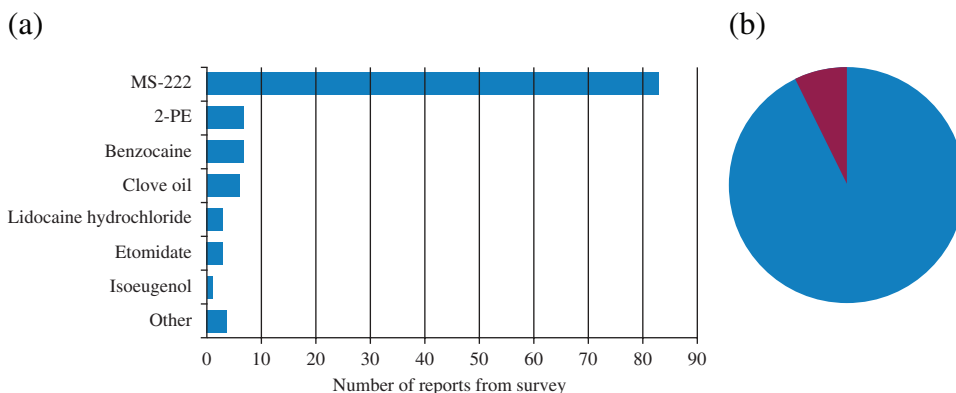


FIG. 2. (a) The frequency distribution from the *Danio rerio* survey responses to the question, 'What anaesthetic agent do you routinely use?' and (b) 'If you use MS-222, do you routinely buffer the solution?' Where ■ is yes and ■ is no.

in many laboratories for larvae (42 of 89) and for broodstock (35 of 88). Other agents used for euthanasia included benzocaine, etomidate, 2-phenoxyethanol, and clove oil. Death was not routinely confirmed in 15 (of 88) laboratories, but destruction of the brain (pithing) (20 of 88) and whole body maceration (11 of 88) were common methods, with alternatives such as visual confirmation of death (absence of circulation, gill movement, reaction to external stimuli), prolonged freezing and overdose of MS-222 reported as additional free text responses (Fig. 4).

Almost half of respondents do not carry out any methods of refinement to improve the welfare of *D. rerio* during anaesthesia or euthanasia (48 of 89). Approaches that were used included buffering MS-222, passing oxygenated water through the mouth and across the gills, applying anaesthesia in the dark, adjusting the concentration of anaesthesia and implementing the latest recommendations from veterinary staff and the scientific literature. Several respondents showed an interest in finding more humane approaches to anaesthesia and were investigating alternatives in their own laboratories.

Electrocution is a permitted method in Europe under current legislation (EC, 2010) and widely considered the most humane approach in the European aquaculture industry (Lines & Spence, 2012, 2014). No laboratory, however, reported using electrocution as a method for euthanasia. Methods for larvae also included physical methods such

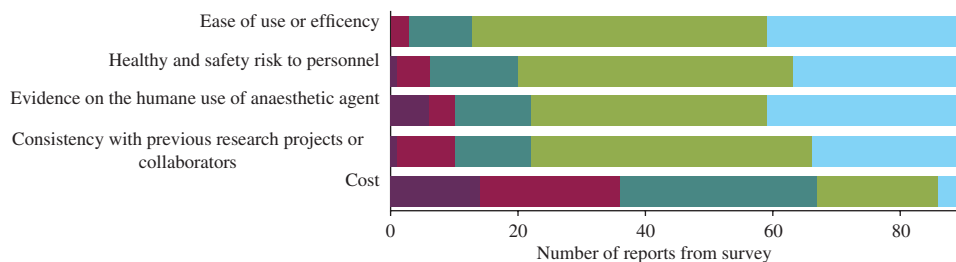


FIG. 3. Frequency distribution from the *Danio rerio* survey responses to the question, 'How do you rate the following in terms of importance when deciding which anaesthetic agent to use?' ■, Not important; ■, slightly important; ■, moderately important; ■, very important; ■, extremely important.

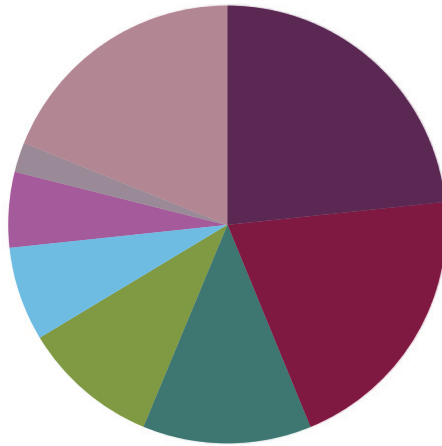


FIG. 4. Frequency distribution from the *Danio rerio* survey responses to the question, 'How is death confirmed?' ■, Destruction of the brain; ■, freezing; ■, whole body maceration; ■, lack of gill movement; ■, decapitation; ■, lack of heartbeat; ■, visual confirmation; ■, death is not confirmed.

as freezing and grinding, but from the questionnaire structure it is not clear if these were used in conjunction with anaesthetics or alone.

HOUSING AND HUSBANDRY

Most *D. rerio* were housed in recirculating systems (58 of 67) that were available from commercial suppliers (49 of 67). Recirculation systems are often chosen from a practical viewpoint when retrofitting aquaria to existing buildings, where large volumes of waste water are not manageable through existing infrastructure. New research groups might, therefore, be expected to begin with recirculation units. Interestingly, 13 (of 67) laboratories used flow-through facilities, which most practitioners would agree is a key method for disease control.

More than half of facilities fed their *D. rerio* three times per day (37 of 67) and most of the remaining facilities were using a two-meal per day strategy (29 of 67). Only five (of 67) respondents used automated feeding and nobody reported using demand feeder techniques, as used in aquaculture. Many facilities used live food daily (47 of 67), but there was a range of extremes with six (of 67) facilities always using live food and two (of 67) who never used it. Live cultures were routinely brine shrimp *Artemia* sp. (58 of 66) and rotifer *Brachionus* sp. (20 of 66). *Danio rerio* were routinely fed laboratory-specific dry food (49 of 67), hobbyist branded dry food (14 of 67) and frozen food (nine of 67).

One aspect of aquaria facilities that is often overlooked is lighting. Transition time from day to night was instant lights-on–lights-off in 45 facilities (of 67), but 18 (of 67) used transition fading, varying in length of time from <10 min to >20 min.

BREEDING AND PRODUCTION

The number of *D. rerio* strains typically held in the facility varied from 15 strains (19 of 77) to 50 or more separate strains (26 of 77). The size and capacity of a facility

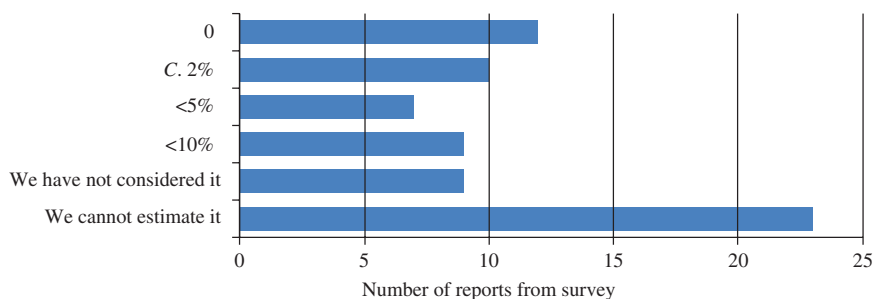


FIG. 5. Frequency distribution from the *Danio rerio* survey responses to the question, 'Approximately what proportion of fish over 5 days old are not used?'.

can probably be most reliably estimated from their capacity for broodfish. The survey results support a cross sectional response when the size of facility is considered. The number of broodfish held ranged from <500 (18 of 76), 500–1000 (9 of 76), 1000–5000 (22 of 76), 5000–10 000 (13 of 76) and $>10\,000$ (14 of 76). The number of staff at each facility of 98 responses, 24 had less than five staff; 31 had five to 10 staff; 21 were 11–20 staff; 20 were 21–100 staff and two had a staff of >100 .

Broodfish were replaced most commonly once per year (43 of 77 responses), but many facilities reported stock replacement twice per year (13 of 77) or three times per year (six of 77). Individual broodfish were spawned either weekly (32 of 77) or fortnightly (21 of 77), but most were housed in mixed-sex stock tanks between planned matings (73 of 77). Typical broodstock tank working volume was 1–51 (35 of 77) or 6–101 (27 of 77) and 10 facilities (of 77) reported using tanks of >101 . Stocking density of adult *D. rerio* was typically 1–5 individuals l^{-1} (53 of 77) or >5 individuals l^{-1} (17 of 77), which probably reflects the prevalence of the commercially available systems, but two facilities (of 77) reported low stocking densities $<1 D. rerio l^{-1}$.

The typical ratio of males to females for spawning was 1:1 (39 of 77) or 1:2 (17 of 77), but six facilities (of 77) reported that they routinely used more males than females. Pedigree was tracked over generations for 54 (of 77) laboratories.

Twenty-three of 76 of survey respondents were unable to estimate the proportion of *D. rerio* >5 days old that are not used (Fig. 5). A further nine stated that they had not considered it. In contrast, 22 (of 76) laboratories were able to maintain overproduction below 2% demonstrating clearly well managed facilities.

REFINEMENT OPPORTUNITIES

To gain an understanding of the current landscape of refinement approaches to improve the welfare of *D. rerio*, survey respondents were asked what refinement approaches they used [Fig. 6(a)]. Three examples were suggested that are often considered as opportunities for refining or enriching the experience of fishes: live prey feeding to allow fishes to express natural feeding behaviours; physical enrichment in the tank, such as plants and gravel that offers a more complex environment; aeration of the water to provide movement and encourage exercise (Williams *et al.*, 2009). Refinement approaches currently used included providing live food (85 of 98). This is a lower proportional response than to the same question asked of the technical staff alone, where only two of 67 reported they did not use live food. In this part of the

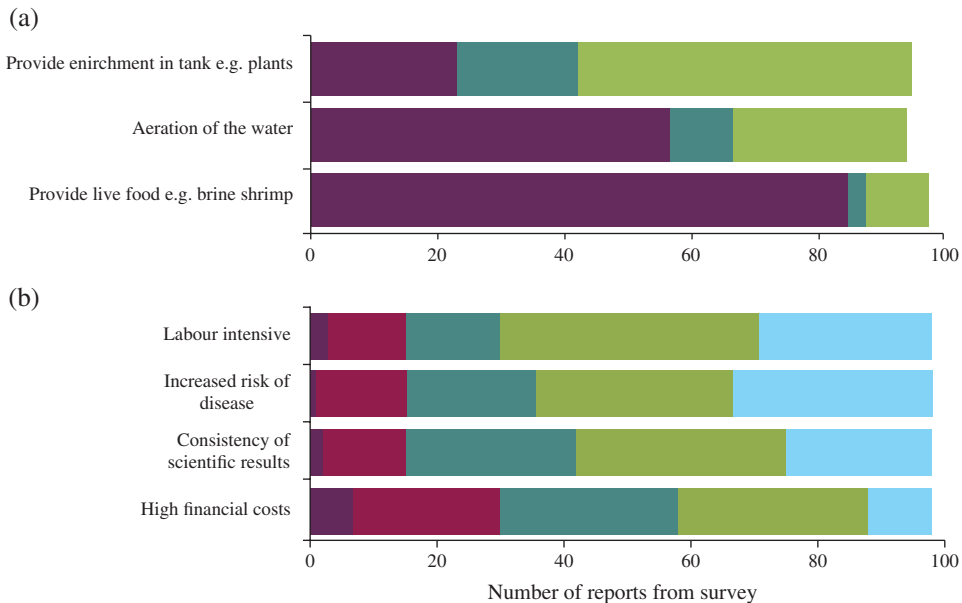


FIG. 6. Frequency distribution from the *Danio rerio* survey responses to the questions, (a) 'What refinement opportunities, if any, do you use to improve the welfare of *D. rerio* in research?' (dark purple, Refinements currently in use; teal, preferred refinement; light green, refinement not acceptable). (b) 'Do you consider the following to be challenges for providing refinement opportunities in *D. rerio*?' (dark purple, strongly disagree; maroon, disagree; teal, neither agree nor disagree; light green, agree; light blue, strongly agree).

survey, 10 of 98 reported they would not consider using live food. The authors did not enquire why this might be the case, but presume it part of a strategy to minimize disease risk (Mason *et al.*, 2016). While it has been suggested that a single diet may be appropriate for *D. rerio* (Lawrence, 2016), the balance between nutritional needs and benefits of being able to display a range of normal feeding behaviours (hunting) are poorly understood. Plants and gravel were not considered an option in 53 of 95 laboratories and only 23 (of 95) reported using them. The authors did not ask if plants were live or artificial. Aeration of water was used in 57 of 94 facilities. The survey did not separate the function of providing oxygenated water from the specifically intended purpose of environmental enrichment *via* water movement.

Other refinement opportunities suggested *via* the free-text questions included approaches to ensure social housing with *D. rerio* or other species. Environmental enrichment approaches included synthetic plants and pictures of gravel under the tanks. The main challenges to providing refinement opportunities included the additional labour required, increased risk of disease, consistency of scientific results and high financial costs [Fig. 6(b)]. There was also a paradoxical concern that adding environmental enrichment to the tank could induce stress and a view that further evidence was required to help inform the best choice for environmental enrichment.

Survey respondents were provided with the opportunity to suggest what was required to overcome these challenges. Suggestions included increased funding of resources and research in this area to create a scientific evidence base, greater education of staff, new technology to allow automated approaches to reduce staff burden, more discussion

between facilities in regards to good practice and an open forum to allow researchers to share refinement experiences.

DISCUSSION

LIMITATIONS OF THE SURVEY

These 98 laboratories probably represent <3% of the total global *D. rerio* community and whilst a larger sample would be beneficial, the findings represent a conservative approach to current good practice. In comparison, the most recent survey on husbandry and health reported views of 19 *D. rerio* laboratories (Lawrence *et al.*, 2016). The current survey represents almost 10% of the ZFIN community (currently *c.* 1000 laboratories). It seems reasonable to infer that the ZFIN community is likely to include those laboratories with significant effort in the area of refinement and so presumably represent the range of current good practice.

ANAESTHESIA AND EUTHANASIA

Anaesthesia is widely used by the *D. rerio* community (89 of 98 respondents) for routine husbandry, such as to aid acquisition of fin clips for genotyping. This clearly represents large numbers of *D. rerio* exposed to anaesthetics. The authors suggest that fin-clipping is an area that could offer the opportunity for refinement if genotyping might be completed without the invasive fin-clip and recently this has been reported (Breacker *et al.*, 2017). There are efforts reported in the scientific community to refine this procedure in other species such as *Neolamprologus pulcher* (Trewavas & Poll 1952) (Le Vin *et al.*, 2011) and *Gasterosteus aculeatus* L. 1758 (Sebire *et al.*, 2015). Handling the smaller *D. rerio* without anaesthesia, however, may prove stressful.

The majority (83 of 89) of survey respondents routinely use MS-222 as an anaesthetic agent [Fig. 2(a)]. Recent emergence of evidence showing MS-222 may be aversive to *D. rerio* and not the most humane anaesthetic choice (Readman *et al.*, 2013; Wong *et al.*, 2014) may be one driver for the reports that other agents were also in use. Clearly, several laboratories are using multiple agents as there were 31 responses reporting agents such as benzocaine, clove oil, 2-phenoxyethanol, etomidate, iso Eugenol, lidocaine and others in addition to the MS-222 responses (114 responses from 89 respondents) [Fig. 2(a)]. In many regions, MS-222 is the only legally permitted anaesthetic for fishes (not just *D. rerio*); clearly this area is one of interest (Anon, 2014). Given that more than half of respondents observe adverse effects when using anaesthesia, these findings lend weight to the urgent need for further work to establish the most humane approach. Most laboratories are aware of the pH effects of MS-222 and buffer accordingly, but pH cannot explain all of the observed adverse responses in *D. rerio* [Fig. 2(b)] and buffering may not be critical in practice within the media used by many laboratories (Wilson *et al.*, 2009). The authors did not ask for the range of concentrations used for the various anaesthetic agents.

Hypothermal shock (plunging into ice-cold water) was a widely used method of euthanasia for larvae (42 of 89) and adults (35 of 88). In Europe this is not a permitted routine method, but it is in many other world regions and is reflected in this global survey. It appears from the literature that there is relatively little evidence to support

the humaneness of the hypothermal shock method. For example, adults show signs of distress in 39% of observations, but this is balanced against 100% of *D. rerio* in both buffered and unbuffered MS-222 that show similar aversion (Wilson *et al.*, 2009). Furthermore, larval *D. rerio* (14 days old) may have a viable blood circulation for 40 min in ice slurry (Strykowski & Schech, 2015). The definitive endpoint study for *D. rerio* euthanasia by hypothermal shock is yet to be reported in the literature (the time for brain disruption on contact with ice cold water *via* electro-encephalogram). Importantly, best practice in the aquaculture industry has moved away from ice as a method of humane slaughter based on ethical concerns; their situation, however, is generally with large, relatively cold-water species, not small neo-tropical species like *D. rerio* (van De Vis *et al.*, 2003; Conte, 2004; Håstein *et al.*, 2005; Poli *et al.*, 2005).

Euthanasia is a complex process to be conducted humanely (Hawkins *et al.*, 2016). Anaesthesia overdose is not necessarily successful for all *D. rerio* ontogeny; larvae have been observed to recover from extended periods of exposure (Wilson *et al.*, 2009). Strykowski & Schech (2015) report 14 day old *D. rerio* exposed to $900 \mu\text{g l}^{-1}$ MS-222 do demonstrate cessation of heart beat after 10 min, but 100% of these *D. rerio* recovered on transfer to fresh water. Many laboratories conduct euthanasia as a two-step process: render the animal senseless, then confirm death *via* a second step such as a physical destruction of the brain. This is the required process under legislation in Europe (EC, 2010). Death was not routinely confirmed in 15 (of 88) laboratories and clearly this is an important area for these laboratories to review their processes and ensure the humane approach to euthanasia.

Electrocution is the preferred method of humane slaughter in aquaculture, but has received little attention for euthanasia in the laboratory (van De Vis *et al.*, 2003; Håstein *et al.*, 2005). No respondents in the survey use the method for *D. rerio*. With more than half of respondents reporting adverse observations with the anaesthetic approach, there appears to be clear justification for a global review of the evidence for good practice anaesthesia and euthanasia in *D. rerio*, particularly given the significant numbers of animals involved.

HOUSING AND HUSBANDRY

Feeding represents a significant investment in staff time, is an opportunity for monitoring of *D. rerio* health and welfare and a route to increase environmental enrichment of these animals (Williams *et al.*, 2009). Automated and demand feeders were not popular, possibly because they reduce human contact, or perhaps other practical reasons. With most laboratories feeding several times per day (and this survey reports more frequent feeding than Lawrence *et al.*, 2016) and a range of dry laboratory diet, frozen food and live prey, the variation in food quality and quantity is clearly significant across the community. Feeding may well be the most critical area for refinement of *D. rerio* care and use in order to standardize the approach and reduce variation both within and between laboratories (Lawrence, 2016; Watts *et al.*, 2016). Indeed, relatively little is known about the optimum diet for *D. rerio* in the laboratory (Watts *et al.*, 2016) and there is a clear opportunity for work in this area to have significant benefits for *D. rerio* welfare.

The authors chose not to ask questions about temperature and water chemistry (*e.g.* pH, oxygen, ammonia, nitrite, nitrate and hardness) as these are largely reported in the literature within each study and an appropriate overview is now available in Lawrence

et al. (2016). Respondents were asked, however, about the typical size of tank and stocking densities as these are likely to affect welfare, yet little is known about current practice or indeed the evidence base for this. Clearly, the conditions provided in each laboratory are *de facto* currently successful for raising *D. rerio* and conducting the science. It seems that most laboratories use stocking densities in excess of one adult *D. rerio* l⁻¹ and a large proportion house >5 *D. rerio* l⁻¹ (also reported by Lawrence *et al.*, 2016). Two facilities, however, kept *D. rerio* at low density stocking density (<1 l⁻¹). It is suspected these may have general facilities more like some hobbyist aquaria, rather than the intensive high density rack facilities. The size of aquaria and reluctance to use plants and gravel from more than half of the respondents also points to the practical aspects of the commercial rack-type facilities. Definitive work on establishing stocking density appropriate for both production efficiency and welfare is apparently lacking from the scientific literature (Lawrence, 2016) and may be difficult to acquire (Gronquist & Berges, 2013). An inter-laboratory study examining stocking density of 3, 6 and 12 *D. rerio* l⁻¹ has been reported and showed no effect on clutch size, spawning success or viability at these densities; but the effects of lower densities are not known (Castranova *et al.*, 2011).

One aspect of aquaria facilities that is often overlooked is lighting. The ratio of light:dark, and occasionally the light intensity, are reported in the literature (Villamizar *et al.*, 2015), but transition periods, whether instant or phased, are rarely reported. It is a surprise that this aspect of light transition is not more widely considered across the facilities. There is a significant literature that uses the startle response to sudden light transition in *D. rerio* as an experimental tool to assess optokinetic reflex and visual motor behavioural responses (Emran *et al.*, 2008; Portugues & Engert, 2009). A subsequent review of the literature, however, has failed to show significant investigation into the benefits of phased transition of lighting for *D. rerio* (or indeed any fish species). The authors have previously advocated phased light transition (Williams *et al.*, 2009) and anecdotally it seems very common in commercial aquaculture. There appears, however, no clear evidence on which to base the decision either to just switch lights on and off, or to replicate a dawn and dusk period. This represents both an opportunity to establish the science and potentially offer a simple refinement if it can be shown to be of benefit either way.

BREEDING AND PRODUCTION

The survey found variation in the frequency of feeding, including once per day (12%), twice per day (43%) and three times per day (55%). This result supports the view of Lawrence (2016) that feeding regimes are sub-optimal, as the variation across laboratories in terms of feeding frequency and diet are unlikely to be within an optimal range for any species. High feeding rates are a significant risk to recirculation systems that must deal with the high nitrogen excretion without causing harm to the entire system. It seems unlikely that *D. rerio* will be underfed since nutritional stress will reduce breeding capacity and growth, but the overall welfare effect of the current situation remains unknown (Nasiadka & Clark, 2012).

Concerns have been raised about consistency and bacterial risk of live foods (Mason *et al.*, 2016; Watts *et al.*, 2016). *Danio rerio* are not specific pathogen-free laboratory animals; they live in an environment where bacteria and viruses thrive and the value of

live food for some life stages seems critical for success (Williams *et al.*, 2009). Variation in food quality will clearly be difficult to harmonize and little is known about practices from one laboratory to another. For example, newly hatched brine shrimp may be fed directly or actually enriched with high lipoprotein supplements. The nutritional value of rotifers may be enhanced by the species of algae on which they are fed, potentially using them as a delivery mechanism rather than a food source. The authors' experience suggests the opportunity to hunt live prey is of significant welfare benefit to *D. rerio* (Williams *et al.*, 2009), but as with many of the current practices, the evidence is far from clear. There is a clear opportunity for further refinement in this area. Optimization of feeding for life stage and likely strain-specific conditions, will mean that *D. rerio* are more likely to be in the best physiological condition and therefore have the basic position for good welfare.

If it is inferred that this survey represents a reasonable cross section of the zebrafish community, then the conservative average facility holds *c.* 1000 broodfish (typical of 35% of respondents to this survey; 65% had significantly larger stocks). Assuming 1000 broodfish is representative of the 3250 institutes highlighted by Kinth *et al.* (2013), then one might expect 3.25 million brood *D. rerio* are currently held in laboratories worldwide. The results, however, suggest approximately 56% are replaced annually, 17% are replaced every 6 months and 8% every 4 months, suggesting an annual broodstock requirement of 5.135 million adult *D. rerio*. These are not necessarily those entering scientific studies; they are the source of embryos and the survey suggests most of these are spawned weekly. Many of these broodfish will be genetically modified and so may be captured in some national statistics on animal use in science, such as those published in the U.K., but largely this number is unknown globally. In the U.K. in 2015, there were 267 385 genetically modified *D. rerio* bred, but not used in further study (Home Office, 2016). It can be presumed these are broodfish, but this figure does not include those without genetic modification. These are not required to be reported suggesting broodstock are a far higher number in the U.K. In the U.K. (and Europe), *D. rerio* that are older than 5 days post fertilization are protected in the laboratory under law. To recognize the importance of broodstock it is interesting to note that in the U.K., genetically modified *D. rerio* alone represent more than those that are reported to have entered studies (147 760) (Home Office, 2016). This would suggest that these broodfish are used for generating significant numbers of larvae for study before they reach 5 days post fertilization (the point they become legally protected and reported in Europe) and therefore the number of *D. rerio* used in science remains unknown.

OPPORTUNITIES FOR REFINEMENT

The current landscape of refinement approaches to improve the welfare of *D. rerio* appears varied [Fig. 6(a)]. Environmental enrichment for fishes is probably most associated with the addition of natural items such as plants and gravel. The benefits of these are difficult to establish for *D. rerio* (Wilkes *et al.*, 2012; Schroeder *et al.*, 2014) and other species [*e.g.* in goldfish *Carassius auratus* (L. 1758) (Sullivan *et al.*, 2016)]. It may be these are of greatest benefit during breeding (Collimore *et al.*, 2015; Wafer *et al.*, 2016). Given the nature of the commercial rack-style facilities, it is unsurprising that plants and gravel were not considered an option in more than half the laboratories, probably on a practical basis. The respondents reported challenges to providing

refinement opportunities that included the additional labour required, increased risk of disease, consistency of scientific results and high financial costs [Fig. 6(b)]. From the authors' previous experience (Williams *et al.*, 2009), information about opportunities for refining or enriching the fish's experience was requested. Feeding live prey to allow *D. rerio* to express natural feeding behaviours was popular in most facilities, although the authors did not ask if this was the specific purpose, so nutrition could be the primary driver. The evidence for the welfare benefit of exhibiting hunting behaviour as opposed to nutritional benefits has not been addressed for *D. rerio*. Exercise and ability to play in areas of water movement are also advocated by some as of enrichment potential and aeration of the water to provide movement is a method to do this (Williams *et al.*, 2009). Whilst aeration was used in more than half of facilities, the primary purpose of enrichment or oxygenation is not clear.

The respondents weighted their response of importance to factors that are seen as challenges to implementing enrichment [Fig. 6(b)], but they also identified what was required to overcome these challenges in free text. Probably, the most important included increased research in this area to create a scientific evidence base.

In short, a total of 98 survey responses were received from laboratories in 22 countries in Europe, North America, South America, Asia and Australia. The range of facility size was broad with approximately equal representation for rank by number of broodfish (<500, <1000, <5000, <10 000 and >10 000). Over half of respondents (61 of 98) reported observing some adverse effects when using anaesthesia. Anaesthesia was a first step of euthanasia in most (63 of 89) respondent's laboratories, but death *via* a second step was not routinely confirmed in 15 (of 88). Euthanasia was achieved primarily by MS-222 but other methods such as iced water were commonly used for both broodstock (35 of 88) and larvae (42 of 89). Most *D. rerio* were housed in recirculating systems (58 of 67) that were commercially available (49 of 67). Few facilities used phased light transitions; instant lights on/off was used in 45 (of 67) facilities, which could be an area for future refinement to improve welfare. Respondents suggest most adults were stocked at 1–5 individuals l⁻¹ (53 of 77), spawned weekly (32 of 77), and replaced annually (43 of 77). Nineteen (of 77) laboratories, however, replaced their broodfish at least every 6 months, suggesting the current broodstock population could be 5 million per year globally. It appears that 22 (of 76) laboratories keep overproduction below 2%, but 32 (of 76) either had not considered nor could estimate the proportion of *D. rerio* over 5 days old that were unused; a potential opportunity for reducing wastage of *D. rerio*. Simple methods for environmental enrichment, such as plants and gravel, were not considered usable by 53 (of 95) laboratories, but 85 (of 95) already use live food. Overall, whilst many laboratories were clearly working towards high welfare standards, there appears to be some opportunity for improvement in the community.

COMMENTARY AND OPPORTUNITIES

This survey provides an overview of the international approach to *D. rerio* welfare and husbandry in 2016. There are clear concerns and opportunities for the community to work together to further improve the welfare of these important laboratory models. They include anaesthesia, where there is a clear and urgent need to identify the most humane methods of anaesthesia and euthanasia. Widespread observation of aversive responses to MS-222 raises concerns about the use of this compound for *D. rerio*,

especially given that it is the only legal option in some countries. Euthanasia, where use of ice (hypothermal shock) is widespread and whilst a legally permitted routine method in many world regions, the evidence base for humane efficacy is underdeveloped. Under European legislation, euthanasia is a two-step process where the fish is first rendered senseless and then death is confirmed by a second method (e.g. anaesthesia followed by destruction). This approach is not universal and care should be taken in all laboratories that *D. rerio* are unable to recover from anaesthesia before death. Feeding optimization (and potentially standardization) is an area for further investigation. Given that diet and body condition can have such profound effects on results of experiments, differences in practice could have significant scientific implications. Lighting, particularly the transition between dark and light appears to represent an opportunity to establish the science behind the need for a phased transition compared with lights-on–lights-off. Intuitively one would assume a simulated sunrise and sunset would probably mitigate any startle response, but in practice the extent of habituation is not known. Environmental enrichment, such as plants and gravel (and probably other items in the tanks that make the environment more complex) are not considered practical by many laboratories. The true value and benefits need to be established and communicated. Fin clipping for genetic identification is common among laboratories. There appears an opportunity to develop less invasive methods and refine this process of anaesthesia and tissue collection. Overproduction is a concern both from ethical and financial viewpoints. It is reassuring that many laboratories have taken this area seriously and are confident in managing their resources such that fewer than 2% of *D. rerio* are not utilized. Clearly, there are opportunities for well-designed, systematic reviews to fill gaps in the evidence base for appropriate anaesthesia; euthanasia; nutrition; stocking density; lighting; and live foods. Research funding opportunities are available to provide an evidence base to address these data gaps such as the NC3Rs funding schemes (www.nc3rs.org.uk/funding).

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Supporting Information

Supporting Information may be found in the online version of this paper:
Appendix SI. Survey on the use of zebrafish *Danio rerio* in research.

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