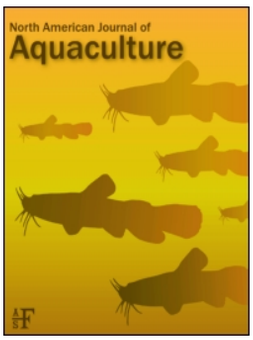

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TECHNICAL NOTE

Standardized Assessment of Thin-film Vitrification for Aquatic Species

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Abstract

Ultrarapid cooling under the appropriate conditions will produce vitrification, a glass-like state used to cryopreserve small sample volumes, but there are a number of major technical drawbacks impeding the application of vitrification to germplasm of aquatic species. These include a lack of suitable devices, and poor reproducibility and comparability among studies due to a lack of standardization. We used 3-D printing to produce a viewing pedestal coupled with a classification system to rapidly assess frozen film quality of vitrification loops. Classification time declined with practice from 2.1 ± 0.3 sec (mean ± SD) to 1.5 ± 0.2 sec (after 200 assessments), and assessments were consistently made in < 2.5 sec. Classifications should be reported with representative images allowing harmonization for quality control. This approach permits rapid classification and can be applied for the development of methods including the evaluation of vitrification solution components, concentrations of solutions and target cells, and configurations and volumes of new devices. Future studies should address the custom fabrication of 3-D printed vitrification devices for use with aquatic species and other applications.

Vitrification has become increasingly viewed as an alternative to conventional cryopreservation for sperm of aquatic organisms and has been studied in at least 11 species of freshwater and marine fish (Kása et al. 2017). Based on ultrarapid cooling of small volumes, vitrification forms amorphous ice known as glass, which circumvents most of the problems associated with cell damage caused by ice crystal formation (Yavin and Arav 2007). The small volumes involved (2–30 µl) limit utility in applications with large-bodied fishes that can produce thousands of eggs but becomes an advantage when working with small-bodied fishes that can produce 10–100 eggs per female or with males that produce minuscule sperm volumes, such as in sex-reversed Southern Flounder *Paralichthys lethostigma* (Hu et al. 2016). Small fishes include ornamental aquarium species and important biomedical models, such as Japanese Medaka *Oryzias latipes, Xiphophorus* spp., and Zebrafish *Danio rerio* (Yang and Tiersch 2009). Small fishes are also disproportionately observed on lists of imperiled species and are in need of germplasm repositories for conservation (Cuevas-Uribe et al. 2011a, 2011b). In addition, vitrification does not require specialized equipment and can be done in remote locations, such as in the field where endangered fishes occur in the wild. Vitrification is also viewed as a viable method to address cryopreservation of eggs and embryos of aquatic species, which is not possible at this time through conventional cryopreservation (Hagedorn et al. 2002).

However, there are a number of major technical drawbacks impeding the application of vitrification to fish and shellfish. First is the lack of devices specifically designed for use with aquatic species, forcing researchers to experiment with devices designed for other uses, such as vitrification of human embryos (Kása et al. 2017). Other options are to use devices not designed for vitrification, such as microbiology inoculation loops (Kuwayama 2007; Cuevas-Uribe et al. 2011b). In addition, there are a large number of technical variables that are not standardized in this research, making it

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problematic or impossible to directly compare vitrification results among studies. Novel fabrication technologies such as 3-D printing offer the potential to design vitrification devices for aquatic species, thereby addressing the first problem but potentially compounding the second problem, the lack of standardization.

For that reason, our approach was to combine a new device with appropriate practices. Our goal was to design a method for rapid assessment of vitrification by classification of the clarity of frozen thin films suitable for use in studies with aquatic species. To do this, we used 3-D printing to fabricate a device that can be used to process vitrification loops and provide rapid classification of vitrification status. This device was a viewing pedestal with parallel reference lines coupled with a practical classification system. Our objectives were to (1) design and prototype by 3-D printing a working pedestal for classifying thin-film clarity, (2) establish categorization criteria as a procedure, and (3) test and optimize the device and procedure for efficiency, including time for use. This approach can be applied for the development of new methods, including the evaluation of vitrification solution components (Yavin and Arav 2007), concentrations of solution ingredients and target cells, and configurations and volumes of new devices such as 3-D printed loops (Tiersch and Monroe 2016).

METHODS

Designing and printing.—Computer-aided drafting (CAD) software (Autodesk Inventor Professional 2016, student edition; San Rafael, California) was used to create a 3-D design of the assessment pedestal (Figure 1). Multiple designs were exported from the CAD software as stereolithography files and opened into the MakerBot printing application. The pedestal was printed with a MakerBot 3-D printer (Replicator2, New York) using polylactic acid thermoplastic filament (MakerBot Industries, One MetroTech Center, New York). The polylactic acid filament was fed through the printer head, heated to a molten state (200°C), and extruded to harden in 0.2-mm layers on a modeling plate (Bartolomé et al. 2017). Our design criteria were (1) a raised pedestal for support, (2) proper positioning for quick alignment on the device, (3) rapid classification of samples, (4) straightforward but relevant classification categories, and (5) the ability to provide standardization among user groups.

Initial prototyping focused on a variety of pedestal designs before we selected an 83-mm-tall pedestal with a round base (62 mm in diameter). The top of the pedestal was produced at a 65° angle with a 5-mm central groove that was used to rapidly orient the axis of the vitrification device (basically a long handle with a small loop at one end) for viewing (Figure 1). When positioned, the terminal loops containing frozen films could be viewed in relation to three perpendicular horizontal reference lines positioned along a viewing panel. The three reference lines were 2-dimensionally printed parallel to one another (Georgia font, bold, 8 point) onto a sheet of standard white computer paper (X-9 multipurpose paper, 75 g/m^2; Boise Paper, Boise, Idaho). The reference lines were spaced 1.5 mm apart to accommodate for vitrification loop length. The paper was cut to produce a 30-mm × 22-mm rectangle that was attached to the top of the pedestal with rubber cement (to allow replacement after repeated use). The viewing panel was recessed below the positioning groove and also angled at 65° to assist with the alignment of samples and avoid repetitive motion problems (OSHA 2011).

Initial testing of classifications and inoculation loops.—The classification system was developed to provide categories that covered the range of possible results for frozen samples. The vitrification devices used to develop the classifications (Figure 2) were 10-µl polystyrene microbiological inoculation loops (Nunc, Roskilde, Denmark). These vitrification devices were used in previous studies of vitrification of fish sperm (Cuevas-Uribe et al. 2011a). The loops were dipped into vitrification solution and plunged into liquid nitrogen for cooling and storage (1–24 h). The inoculation loops were removed from the liquid nitrogen and immediately positioned on the pedestal for quality assessment. The viewing panel was recessed 7 mm lower and parallel to the positioning grove so the frozen samples themselves were not in contact with the pedestal. To develop a full range of constraints to test the classifications (described below), several solutions were initially used: deionized water (typically producing category 0, fractured films, or film failures), 50% deionized water plus 50% glycerol (typically category 1), and 100% glycerol (category 2).

Categories were defined to determine vitrification quality, with classifications according to the clarity of frozen films (Figure 2). The degree of clarity was estimated by viewing the parallel horizontal lines on the pedestal through the films. Three categories of clarity received a classification number, labeled as category 0 (opaque or abundant crystalline ice formation), category 1 (translucent or partial vitrification), or category 2 (transparent, substantial vitrification, or glass). Other documented categories were cases of fractured films or film failures. Fractured films disabled the ability to properly assign a vitrification classification and presented opaque cracks, although partial vitrification sometimes occurred in translucent portions between cracks. Failures occurred when films were unable to remain intact during the freezing process.

Evaluation of pedestal and classification system.—Hanks' balanced salt solution was used as an extender component at an osmolality of 300 mOsmol/kg (0.137 M NaCl, 5.4 mM KCl, 1.3 mM CaC_{l2}, 1.0 mM $MgSO_4$, 0.25 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 4.2 mM $NaHCO_3$, and 5.55 mM glucose; pH 7.2). This salt solution is commonly used for fish sperm cryopreservation and has been used in previous vitrification studies. It was used to prepare a vitrification solution (20%

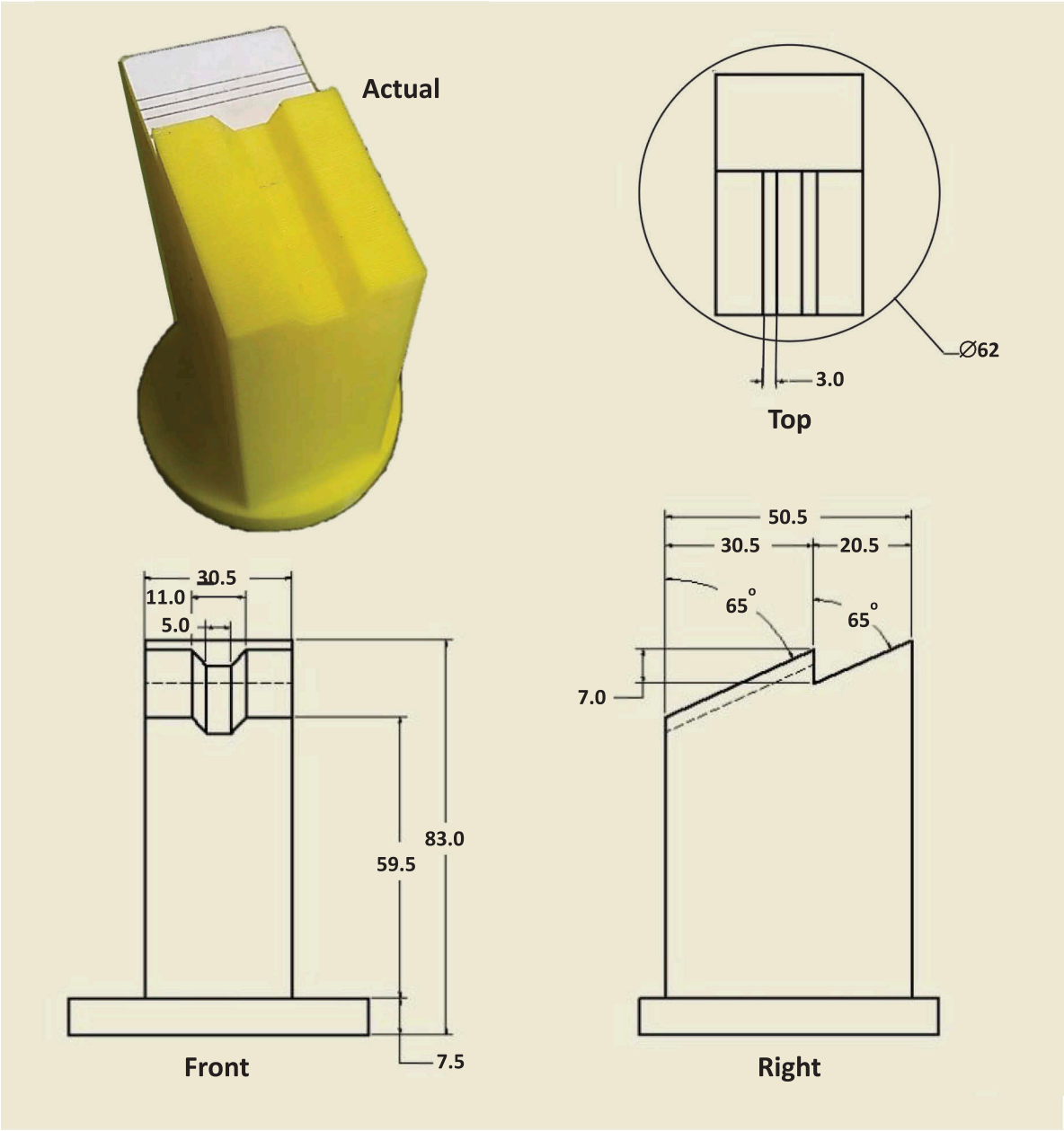

FIGURE 1. Schematic diagram of the viewing pedestal dimensions (mm). The upper left picture shows the actual device after use for 250 assessments.

1,2-propanediol + 20% 2-methoxyethanol + 20% Hanks' balanced salt solution + 40% methanol), which was used in prior research with fish sperm (Cuevas-Uribe et al. 2011a), to test the utility of the assessment system. Two people (assessor and recorder) were used to conduct experimentation. Six batches of 36 loaded devices were submerged individually into liquid nitrogen, where they were held for at least 1 h.

Assessments were made as the recorder started a timer when the assessor said "start," which signified the removal of a vitrification device from liquid nitrogen. The assessor placed the device on the pedestal and voiced a classification. The recorder immediately stopped the timer and documented the time of assessment and the classification. The time between when the assessor removed a device from liquid nitrogen until voicing the classification was the documented time of assessment. Samples were assessed in a walk-in refrigerated room, which remained at 4.5–7.2°C with 65–70% relative humidity. Vitrification solutions typically comprise chemicals that thaw rapidly (Yavin and Arav 2007); therefore, methods used to classify the quality of vitrified films must be performed rapidly. A maximum time for assessment was set at ≤ 2.5 sec. This requirement

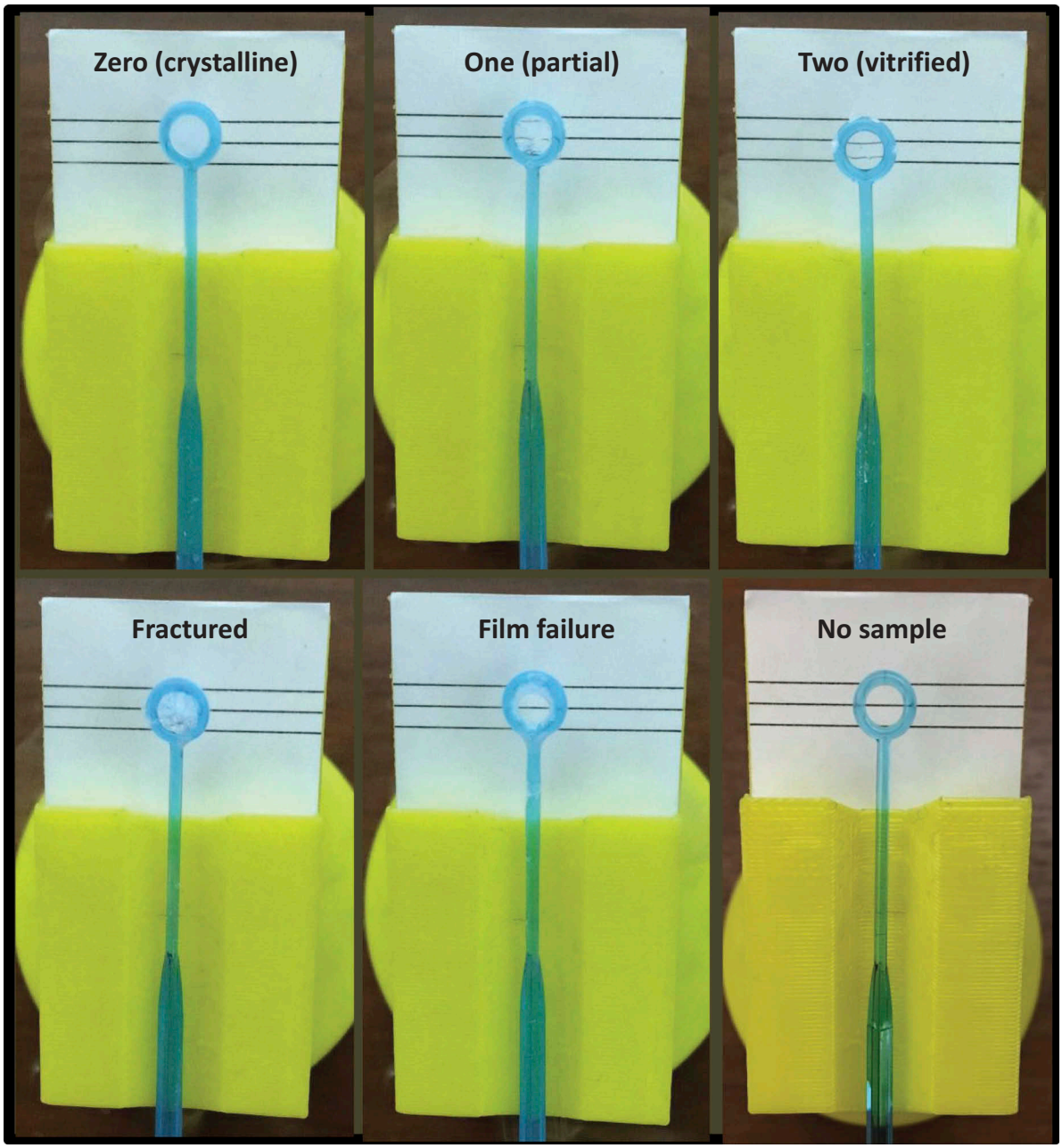

FIGURE 2. Viewing pedestals with representative images of different films in the loops to demonstrate the assessment classifications. The external diameter of the loops is 6.5 mm.

was assigned as the target maximum assessment time, which was considered to be sufficient to ensure that classifications were assigned before the films began to thaw. The implementation of a target maximum assessment time allowed total assessment time per batch to be more consistent than using a total handling time.

Data were analyzed using a general linear model (GLM procedure; SAS 9.4), with Tukey's honestly significant difference test used for differences in assessment times. The level of significance was set for values of $P < 0.05$.

RESULTS

Rapid throughput is essential to efficiently classify large quantities of samples. The overall throughput increased throughout experimentation which consisted of six batches, each comprised of 36 samples. Total handling times for the separate batches varied throughout testing due to the absence of a time requirement for this. Individual assessment times were averaged for each batch and the value declined 0.7 sec throughout experimentation; however, individual assessment times deviated by 0.2–0.3 sec/sample (Table 1). Actual

TABLE 1. The mean ± SD assessment time required per sample was calculated for each batch (made up of 36 samples). The total time (recorded for each batch) consisted of the combined time required to assess and handle the samples; assessment and handling were calculated as the actual times and the percentages of the total times. Mean ± SD assessment times sharing a letter were not significantly different. A maximum target time was set for assessments (≤2.5 sec/sample) but not for handling time.

			Minutes required (% of total time)	
Batch number	Mean ± SD assessment time per sample (sec)	Total time (min)	Assessment	Handling
1	2.1 ± 0.3 z	32	20 (63)	12 (37)
2	1.9 ± 0.2 y	17	11 (65)	6 (35)
3	1.9 ± 0.2 y	25	14 (56)	11 (44)
4	1.8 ± 0.2 y	20	11 (55)	9 (45)
5	1.4 ± 0.2 x	24	11 (46)	13 (54)
6	1.5 ± 0.2 x	20	14 (70)	6 (30)

assessment times declined steadily with experience (Figure 3), as the time to assess individual samples dropped 1.35 sec with experience from the longest to the shortest assessment times ($P < 0.001$; Table 1).

DISCUSSION

Although two people were used to develop and evaluate our classification system, a single person would be adequate to assign classifications in routine applications. Standardized approaches to classification could employ the use of captured videos or images to document visual records of frozen films that could be examined after experimentation, thus providing an accurate archive of classifications and corresponding assessment times. Regardless of the method used for assessment, experience with the assessment device is essential to reliably work within a desired time frame. To optimize use, assessors should gain experience prior to using the device for conducting experimentation.

The 3-D printed pedestal we describe is a visual method of classification. Categories of frozen film classifications are subjective when measured visually. Variables such as device design, frozen film quality, sample clarity, and solution thaw rates currently prevent the establishment of a universal classification system. This impedes research and makes comparisons across studies problematic or misleading. Therefore, as a standardized approach to assist with the communication of results, users should define classifications with representative pictures in publications

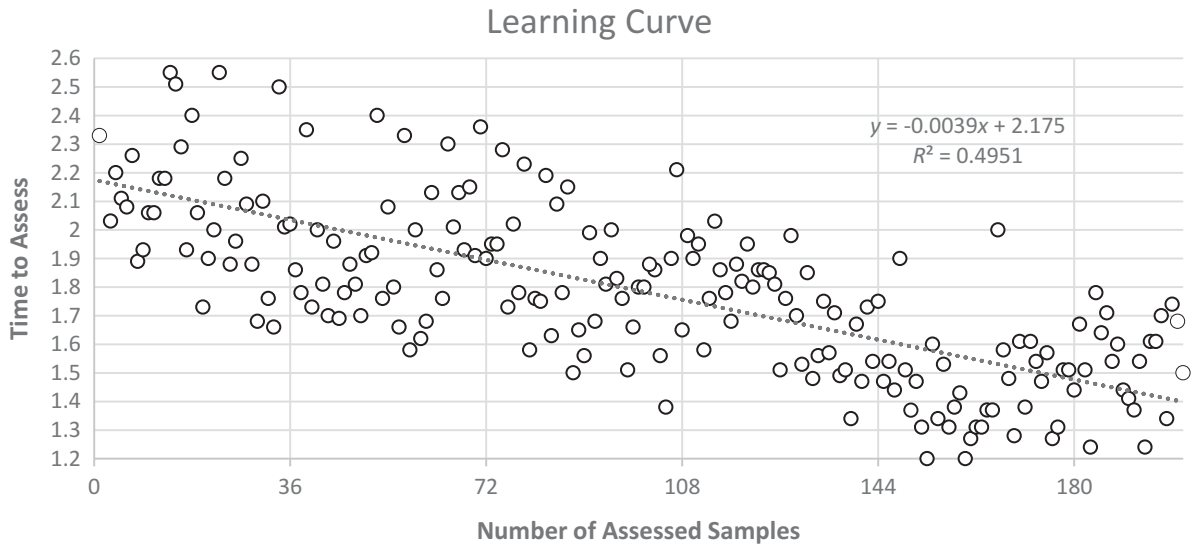

FIGURE 3. The time required to individually assess samples (in sec) declined throughout experimentation, representing a learning curve. Three assessments exceeded the target maximum assessment time of 2.5 sec, all of which were recorded within the initial 50 samples. Film failures (16) were excluded from the 200 assessment times shown (there were a total of 216 assessments).

allowing harmonization of results for quality control (Torres et al. 2016). In addition, groups using aquatic species (such as biomedical researchers or conservation biologists) should address questions of standardization as communities rather than as individual research groups, as is done currently (Tiersch 2011). The application of 3-D printing technology permits the rapid visual classification of vitrification quality within loops. Future studies should address the fabrication of 3-D printed vitrification devices to specifically customize them for use with aquatic species and in other applications. The use of 3-D printing with polylactic acid filament is especially suitable for prototyping and fabrication of devices intended for cryogenic applications (Tiersch and Monroe 2016) and offers tremendous opportunity to improve protocols and to assist in the development of community-based standards due to the increasingly widespread ability to 3-D print design files that are shared over the internet (Hu et al. 2017).

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