Modification of clinical dental impression methods to obtain dental traits from living and whole non-mammalian vertebrates

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Abstract

Dental impressions are routinely in human and veterinary clinical settings to capture dental characteristics three dimensionally for the evaluation of pathology, morphology, eruption patterns, and mastication function. While these aspects of dentition are of great interest to evolutionary biologists, ecologists, and biomechanics researchers, impressions have rarely been used in studies of non-mammalian vertebrates (e.g., fishes) and/or for non-medical research. Studies of animal dentition usually require euthanasia and specimen dissection, low-resolution CT scans and impressions or extractions of individual teeth for study. These practices prevent in-vivo longitudinal studies that factors growth and other chronological changes. For example, it is possible to infer feeding ecology by scanning or observing the surface of teeth and quantifying micrometer-scale topographical abrasions caused by feeding behavior. It is also possible to track tooth replacement or changes in occlusion through time. Here, we describe a method for dental impression that can preserve the life of the vertebrate specimen, be used non-destructively with museum specimens, and has a wide range of applications in organismal research. We demonstrate this method using living specimens of *Polypterus senegalus*, a non-teleost fish (Actinopterygii), in a laboratory setting.

Keywords: *dentition, Polypterus, fishes, morphology,* dental impression, in vivo

Introduction

Vertebrate dentition contains key traits related to diet and feeding behaviors, and therefore can provide essential information on the ecology and evolution of species. In fishes alone, evolutionary and ecological studies of dentition have involved wide-ranging topics such as development (Hung et al., 2015), replacement (Carr et al., 2021; Stuart et al., 2021), dental microwear analysis (Baines et al., 2014; McLennan and Purnell, 2021; Purnell et al., 2006; Purnell and Darras, 2015; Weber et al., 2021), jaw evolution (Ronco et al., 2021), feeding characteristics (Streit et al., 2015), biomechanics and bite force (Deng et al., 2022) and diversification of the oral apparatus (Burress, 2016). However, in all these above example studies, either dead fish and isolated tooth samples were used (Burress, 2016; Streit et al., 2015; Stuart et al., 2021), and often euthanasia was necessary to conduct the experiment (Baines et al., 2014; Hung et al., 2015; Purnell et al., 2006; Ronco et al., 2021). Furthermore, dissection of the jaw or removal of teeth is usually performed, thus permanently deforming the original sample.

Vertebrate biologists have developed a keen interest in methods which are able to derive detailed, high resolution morphological information in non-destructive ways. In the last decade, scanning methods such as optical scanning, micro-CT, laser scanning, and synchrotron use have been increasingly used to obtain skeletal traits from full specimens for various applications such as the creation of trait datasets and biomechanical models (Akhter and Recker, 2021). However, scanning approaches have many constraints which limit their utility in many vertebrates. Optical scanning, as of 2023, lacks the resolution necessary to see fine details such as wear (personal observation). Micro-CT and Synchrotron X-ray scanning imposes size limitations on specimens and have trade-offs between field of view, processing time, and image resolution, and of course take place under high radiation (Larsson et al., 2016; Sun et al., 2022). Usage of X-ray scanning therefore requires dead and or dissected samples in most cases and/or considerable preparations and planning at limited facilities for limited in vivo work to date (Donnelley et al., 2010; Gradl et al., 2018).

Dental impressions offer a way to obtain detailed, high-resolution information from living non-human, especially non-mammal vertebrates, such as fish, including for use with higher resolution imaging such as laser scanning. Simple impression through the use of putty or direct application of material has previously been done on vertebrate specimens, such as fish to observe their dentition and tooth surface (McLennan and Purnell, 2021; Purnell and Darras, 2015; Weber et al., 2021). Nevertheless, its application was limited to fixed dead and skeletal specimens or fallen teeth of living fish, which individual and tooth position are uncertain. For fish and most other vertebrates especially non-mammals without common veterinary dental clinical coverage, there has been almost no practical method to record whole mouth dental information, easily, cheaply, non-destructively, and repeatedly from live and whole specimens.

We propose a noninvasive methodology using 3D printed impression trays and materials adapted from human clinical practice to obtain dental impressions from live

and whole vertebrates. This method is humane and non-destructive, obtains whole-dentition morphological information and occlusion patterns at the same time, and allows for tracing chronological changes essential to understand the tooth replacement rate and pattern or the turnover rate of dental microwear on tooth surface. The method opens the opportunity to study oral function and its temporal change that would not be possible otherwise, and in specimens which are incompatible with other methods of obtaining detailed whole-dentition information. Jaw biomechanics, dental microwear, dental growth, morphology and development are among many areas of research for the potential applications of this method.

Here, we demonstrate the utility and function of the dental tray impression method in non-human vertebrates. Because of ongoing project needs, we developed and tested this methodology on live and preserved specimens of a non-model but increasingly used laboratory organism, the non-teleost fish *Polypterus senegalus*. Below, we describe the details of the method and the results obtained from *Polypterus senegalus.*

Method Details

Impression tray design

In the initial experiment design, a single-arch impression tray was considered. However, due to the sedation time constraints, this would require that each animal undergo separate sessions to take impressions for both maxillary and mandibular dentition. Therefore, single-arch impressions would have necessitated that the animal to be exposed to multiple sessions of potentially stressful general anesthetic procedure (Welker et al., 2007) and within short time intervals, due to the anesthetic effective working time and its similarity to the impression material hardening time (Sawaura et al., 2022).

For the above reason, the use of a dual arch impression tray system (molding both maxillary and mandibular arches simultaneously) was decided (Figure 1A). In addition to maximizing results per sedation session, dual arch design can also increase accuracy and reproducibility of impressions (Johnson et al., 2010; Parker et al., 1997).

For *Polypterus,* the decision to use purpose-designed 3D printed impression trays allowed periodic, fluid, and adaptive modifications of the design to accommodate growth and ensure proper sizing and sufficient capturing of the oral cavity (Figure 1B). A rigid impression tray was also favored as previous studies showed that it increased accuracy of impressions (Carrotte et al., 1998). Taking this into account, the use of poly-lactic acid (PLA) and/or acrylonitrile butadiene styrene (ABS) as the 3D printing material was incorporated into the method.

Tray design was divided into intraoral and extraoral components. Extraoral elements of the tray included a perforated retaining wall that was customized to roughly match the external border and size of the jaw (Figure 1A, right). In designing a suitable tray for this impression, the requirement for firm stabilization of the animal during impression sequence (Figure 1C-F) was a major concern. Voluntary or involuntary sudden movement by animal or operator would play a factor in impression accuracy, air-pocket formation, and impression distortion. Following impression material manufacturer recommendation to limit setting-time movement, extraoral retaining

wall elements to affix and stabilize the head were added. These walls extended posteriorly and past to the angle of the mouth but did not touch the gills. Tray adhesive was not used for ease of retrieving and cutting the impression for microscope observation. Instead, perforations were included in the design of the retaining wall to aid in attaching the impression to the tray. The tray also included a handle, marked to differentiate palatal and mandibular sides, and included current size of the tray.

The intraoral portion of the tray included occlusal stops to avoid dental occlusal distortions and tooth contact with tray during impression (Figure 1A, middle). Incorporating hard or soft tissue stops ensured a uniform impression material thickness (Terry et al., 2010) and minimized the distortion of the impression material (Ortensi and Strocchi, 2000). In *Polypterus*, the centrally located parasphenoid bone on the palatal roof and the tongue (basihyal) in the mandibular floor were designated as hard/soft tissue stops (Allis, 1922). A horseshoe-shaped trench of 0.5-1 mm in depth and uniform width that accommodated the size of the arch was placed on both palatal and mandibular aspects of the tray to allow occlusal clearance for dentition to impress without interference from tray (Figure 1A). This trench followed the external retaining wall leaving the centralized raised parasphenoid stop in its center on palatal section of the tray and a smaller raised central lingual floor on the mandibular side.

The impression tray was designed using Autodesk Fusion360. Trays were 3D printed using Ultimaker S5 (Ultimaker, Netherlands) using Ultimaker PLA and ABS material (Ultimaker, Netherlands). Ultimaker Cura was used to slice the STL files, using the default preset “extra fine” with 0.06 mm layer height. Size was controlled using Cura, to adjust for individual differences in jaw size. We discovered that the tray printed best with the support overhang angle at 80° and brim build plate adhesion type, printing the structure upright with the handle on top and the mouthpiece at the bottom. Depending on the size of the impression trays, 3D printers with less Z axis resolution should also be usable. When larger trays are required, it will be less time-consuming to increase the layer height.

Dentition molding process

The teeth molding process involves a series of steps: 1. Preparation of the tray and impression materials (Figure 2A). 2. Sedation of fish (Figure 2B). 3. Cleaning the teeth using compressed air and primary impression material (Figure 2C-E). 3. Application of secondary impression material to the impression tray and the final impression (Figure 2F-H).

First impression sequence

After confirming the animal has been completely sedated in the temporary sedation tank, it was removed from the water, and tweezers were used to gently pry the mouth open to expose the teeth. Compressed air was used to gently dry the dentition to remove or disperse residual moisture (Figure 2C). This was immediately followed by a double impression process as per previous dental studies involving the use of veterinary dental trays on livestock mammals (Hoffman et al., 2015). First impression was taken with ExaFine Vinyl Polysiloxane Putty Type (GC, Japan) as a cleaning step to remove debris and reduce enamel pellicle (Figure 2D-E). Used latex free nitrile gloves while conducting the experiment to avoid latex inhibition of polymerization of impression material (Baumann, 1995).

Base and catalyst were mixed into a ball or a thick disk using manufacturer mixing directions and placed in the mouth of the sedated individual. Gentle pressure was used to close the mouth on the putty material 2-3 times ensuring that the teeth are fully submerged while the putty was relatively soft. Putty was removed in about 1 minute prior to complete hardening to reduce the risk of enamel damage. For this step, tray use was not necessary.

It has been noted that brushing has a negative impact on enamel surfaces (Wiegand et al., 2007). Factoring the relatively low thickness and different composition of *Polypterus* enamel in comparison to mammalian teeth (Meinke, 1982), this damage can be amplified when using a typical brush. This can be a crucial factor when studying aspects that require enamel surface observation such as dental microwear analysis. Therefore, we decided to use putty to clean teeth surface prior to doing the actual impression instead of brushing which takes less time but might damage the enamel surface.

Final impression sequence

To prepare for the second impression using a more accurate impression material, an Automix tip was affixed to the Dr. Silicon regular (BSA Sakurai, Japan) (Sawaura et al., 2022) cartridge to ensure proper mixing of the dual component Polyvinyl siloxane (PVS). Portion of the impression material was exuded (or bled) prior to filling the impression tray to ensure that only properly mixed material is injected into the impression tray.

To avoid air pocket or bubble trapping, the impression dispenser Automix tip remained submerged inside the impression material as it was ejected. Manufacturer-reported intraoral setting/curing time of 3 minutes and 10 seconds was exceeded to 5 minutes or more during the experiment to compensate for the effect of human intraoral heat that may accelerate polymerization.

The impression tray loaded with medium body PVS was placed into the mouth of the animal and with gentile pressure both jaws were pressed together to simulate the bite (Figure 2G). Animal and impression tray were held together securely yet gently to avoid harm till the impression material was completely set (Figure 2H). The animal is then returned to its original tank and monitored for 2-5 minutes to ensure that it survived the general anesthetic and regained its motor functions. When timed, this two-impression process produced results in about 10-12 minutes. That included sedation time, air drying, mixing, and using silicone putty for the cleaning step, and finally a medium body PVS impression using the customized tray. All fish survived the sedation and impression sequence and successfully repeated the process on subsequent dates.

Scanning of dental impressions

Obtained dental molds were scanned using a confocal laser microscope (VK-X210, Keyence, Japan) with a violet laser (408nm) and an ultra-long range 100x lens. Molds were cut along the line connecting mesio-distal section of each tooth using a scalpel and dental loupes, so that both buccal/labial and lingual/palatal surfaces of teeth can be observed in the impression. The tips of teeth were scanned as this area contact most intensively with food and likely have more dental microwear on the

basis of work with other non-mammal tetrapods (Winkler et al., 2019). The three-dimensional surface models and two-dimensional images were generated using the photo-simulation function of the laser microscope.

Fish husbandry

Polypterus senegalus specimens of 20-25cm size were acquired from local pet shops. 100L water tubs with Eheim Classic 2215 external filters (Eheim, Germany) are used to house up to 2 *Polypterus senegalus* specimens of 20-25cm size. The water temperature is controlled to be 25°C.

Feeding is performed 3 times a week using 2 pellets per fish of Hikari Crest Freak Bottoms (Kyorin, Japan), however any other food for bottom dwelling carnivorous fish should be admissible. 10-20% water change is performed every week, removing detritus and uneaten food. Water quality, including pH, nitrate and nitrite contents are monitored after every feeding, changing 10-20% of water when nitrate and nitrite levels show higher than around 25ppm and 1ppm respectively.

Fish sedation

Tricaine mesylate (MS-222) (Sigma-Aldrich, USA) was used for fish sedation with twice in mass of $NaHCO_3$ (Nacalai Tesque, Japan) as buffer, following previous reported sedation of *Polypterus* (Whitlow et al., 2022). Fish was transferred from the tank to a smaller tank consisting of 2L of water with dissolved buffered MS-222 (Figure 2B). Optimization of fish sedation was performed by gradually changing the MS-222 concentration in increments of 50 mg/L, starting from 100 mg/L, exposing fish to 5 minutes of anesthetics, and measuring the time before the fish starts moving outside the water. We discovered that for the size and species of *Polypterus* specimens we used, 5 minutes of exposure to 300 mg/L of MS-222 was enough to keep the fish sedated for at least 5 minutes outside the water, enough to obtain dental impression. Fish sedation was performed at most once a week.

We observed no mortality on the fishes upon performing the procedure (N=28). As *Polypterus* can breathe air (Magid, 1967), it might be more tolerant to long periods outside of water. However, we only needed 5 minutes to take a mold of an individual, and the actual impression time with blocking of the mouth and pharynx was shorter. If longer time is required, it might be advisable to maintain the level of sedation by running oxygenated water with a lower level of anesthetics through the gills (Neiffer and Stamper, 2009).

Results in *Polypterus*

Using this method, we were able to produce molds that were able to capture accurately the dentition and singular teeth of *Polypterus* (Figure 3A-B). This method was able to accurately capture the intraoral anatomy of the parasphenoid bone with remarkable accuracy (Figure 3A). We scanned the molds taken from different individuals and we were able to effectively capture dental structures less than 1mm (Figure 3C-E) where previous studies struggled (Mihalitsis and Bellwood, 2019). Further, we were able to identify enamel damage on some teeth by quantifying the depths of the ridges on the surface of the teeth (Figure 3F-G).

Discussion

Potential applications

Perhaps the most important application of this method is the ability to produce time series for morphological changes across the entire dentition of living animals, such as tooth damage, growth, replacement, and development (Carr et al., 2021; Streelman et al., 2003; Trapani et al., 2005). Thus, use of this method provides the opportunity for extensive study of the oral apparatus and function while preserving sometimes rare live specimens of non-model, non-domestic species.

Dental impressions can be useful for dental microwear analysis and other applications requiring high impression accuracy for fine details (Hoffman et al., 2015). We recommend a double impression using putty and PVS. However, to observe the dentition and occlusion patterns, performing only one impression using putty may be enough. By only performing one impression, it is possible to reduce stress on the animal by limiting sedation time, and for fish, time out of water.

Dental impressions can record the intricately detailed intraoral soft tissue anatomy of living specimens, including features which could previously only be evaluated on dead specimens (Luczkovich et al., 1995). With the aid of silicone impressions, intraoral structures can be reproduced alongside the teeth using resin or other modeling material (Purnell and Darras, 2015). Physical models made from these impressions can be essential for jaw morphometric analysis studies (Gunter et al., 2013; Wilga and Ferry, 2015). The ability to replicate the jaw margin alongside the tooth impressions provides an alternate avenue to examine bite articulation and enhanced understanding of jaw biomechanics (Westneat, 2003; Wilga and Ferry, 2015). Soft tissue models can also be of use with the physical replication of skeletal movements and feeding mechanics for (XROMM) studies (Camp and Brainerd, 2015).

Although tooth replacement has been extensively studied from a histological and developmental perspective (Huysseune and Witten, 2008) including some work examining fish at various growth stages (Carr et al., 2021; Huysseune et al., 2007) there is very little data on tooth growth and replacement on living individual non-mammals (Carr et al., 2021), with time series from a single individual Recurring creation of whole-dentition tooth records via dental impression provides an opportunity to not only study replacement patterns but also examine external factors (natural and lab fabricated) and their effects in inducing replacement.

Method Limitations and Considerations

Sedation

For this method, the sedation time requires accounting for PVS putty (partial) setting time and medium body PVS (complete) setting time. Changes to the size of fish or other animal might require modification of the sedation method to accommodate time requirement for polymerization time. We found that sedation time can also vary based on individuals and sample needs. We recommend testing sedation concentration and time using at least 3 animals based on best practices.

Different species will require different types of short-term sedation or may not require sedation at all. For larger fishes, or when anesthesia is required to be performed in a

large body of water, it might be impractical to use MS-222. In this case, clove oil can be used as a substitute (Javahery et al., 2012). For other vertebrates, we recommended consulting a veterinarian or best practices for sedation of those species. Maintenance of anesthesia might be required for when more time is needed to take impressions, while optimization of anesthesia concentration needs to be performed depending on species and size.

Some animals, such as domestic mammals (Hoffman et al., 2015) or tetrapod with easy triggered reflexes, may bite a tray willingly without sedation. However, in non-domestic species, creating a detailed impression may require pressing the jaw into the tray or otherwise ensuring a bite with sufficient force on the PVS material or putty.

Impression tray design

Using autoclavable 3D printed material (Fischer and Howell, 2021) would allow for multiple usage of the same tray in the case where sterile trays are required. This might reduce material cost if the type and shape of tray is unchanged throughout the experiment. For smaller individuals with rapid growth rates, creating multiple trays with accommodating sizes might be preferable.

Adding angulation to the floor of the impression tray in a wedge design can redistribute bite forces more evenly in cases where jaw mechanics lack the complexity or range to accommodate the impression tray, (e.g., fish, amphibians).

3D scanning of specimens, where feasible, can be used to help modify the custom trays for other species and individual animals, reducing the need for iterative printing and testing. As noted above, conventional 3D scanning of dentition does have the resolution or needed to identify dental features, such as wear, that are usually seen in micrometer scale. However, the resolution is sufficient for customization.

Mitigating the enamel pellicle and biofilm

For in-vivo dental microwear analyses, pellicle (protein film) formation on tooth enamel between impression sessions was most challenging to manage (Hoffman et al., 2015). Dental biofilm accumulation can interfere with impression accuracy, distorts vital surfaces, and can obscure microwear. The difficulty lies in the abundance of factors adhering the pellicle to the tooth structure and to itself (Boles et al., 2004). For this study it was necessary that we find an efficient, and innocuous approach to remove biofilm prior to taking impressions. This method must also adhere to the time constraints governed by the duration of the sedation process.

Studies have shown that plaque removal is best achieved via irrigation combined with physical debridement (Figuero et al., 2017). In previous dental microwear analyses on larger living animals using directly applied putty, methods used diluted bleach (Sodium hypochlorite) and tooth brush, or a water jet and air drying to prepare the teeth for impressions (Teaford and Glander, 1991). However, for our purposes this method could not be used due to the chemical effects of peroxide on PVS material (Hamalian et al., 2011) and sodium hypochlorite effect on teeth (Sim et al., 2001). On *Polypterus* these effects may be amplified by the small size of the teeth and its delicate enamel structure, in particular collar enamel which can be as thin as 5 µm (Sasagawa et al., 2013). Preservation of enamel structure also prevented any mechanical debridement consideration for this experiment. Therefore,

we decided to perform double impression mentioned above, using putty to clean teeth surface.

Tooth and animal size

The vast majority of PVS dental impression material is designed to accurately replicate the dimensions and details of a human dentition for the purpose of prosthodontic treatment or record acquisition with high reproducibility. In the case of smaller teeth in *Polypterus* and other small animals (.5-1 mm in length), molding success becomes increasingly technique-dependent, and reproducibility more difficult to accomplish. Therefore, it is advisable to obtain impressions from multiple specimens whenever applying this method to new species to confirm reproducibility.

Pharyngeal and extraoral teeth

As the trays used in this method are based on those designed for humans, they are currently only capable of producing impressions of anterior segments of the mouth including the palate and marginal teeth. However, it is possible to produce pharyngeal tooth impressions with individualized customization of the tray to extend intraorally. In addition, precautions must be taken to avoid injury and ingestion of the impression material. Due to the anatomy and lack of deep pharyngeal teeth, that was not a consideration with *Polypterus,* where deep gill morphology was captured alongside the marginal dentition (Figure 3A-B)

Use with fish outside of aquatic environments.

Polypterids, adapted to shallow water and managing hypoxia (Graham et al., 2014), could have an advantage in surviving dry environments and the impression process when compared to other fishes. When applying this method to other species of fishes, physiology can be a factor in determining success and consistency of the results outside water. Fish survivability outside of its environment should be taken into consideration when selecting anesthetic, impression material setting time, and fish husbandry.

Conclusion

Using this dental impression tray method, we were able to consistently obtain dental impressions from live fish and without causing harm to the specimen. We expect that, with some modification, this method can also be applied to other species of vertebrates, such as fishes, where euthanasia was previously required. It has value in analyzing temporal changes in oral and dental morphology, growth, biomechanics, tooth replacement and dental surface in response to diet, feeding ecology and behavior, obtaining data which is not current available for most vertebrates, and fishes in particular.

Data availability

STL files for the impression trays are available on https://github.com/oist/SallanU_impressiontray.

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Author contributions

J.N.W., R.S., T.K., and L.S. conceived the project. J.N.W. and R.S. conceived the method and performed overall direction and planning. J.N.W., T.K., and R.S., performed molding trials. T.K. performed microscope scanning of obtained molds. J.N.W., T.K., R.S. and L.S. wrote the manuscript.

Competing interests

The authors declare no competing interests.

Ethics statement

This research was approved by the ethics committee of the Okinawa Institute of Science and Technology under the protocol number ACUP-2023-006-2.

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Figure legends

Figure 1. Schematic representation of the molding sequence

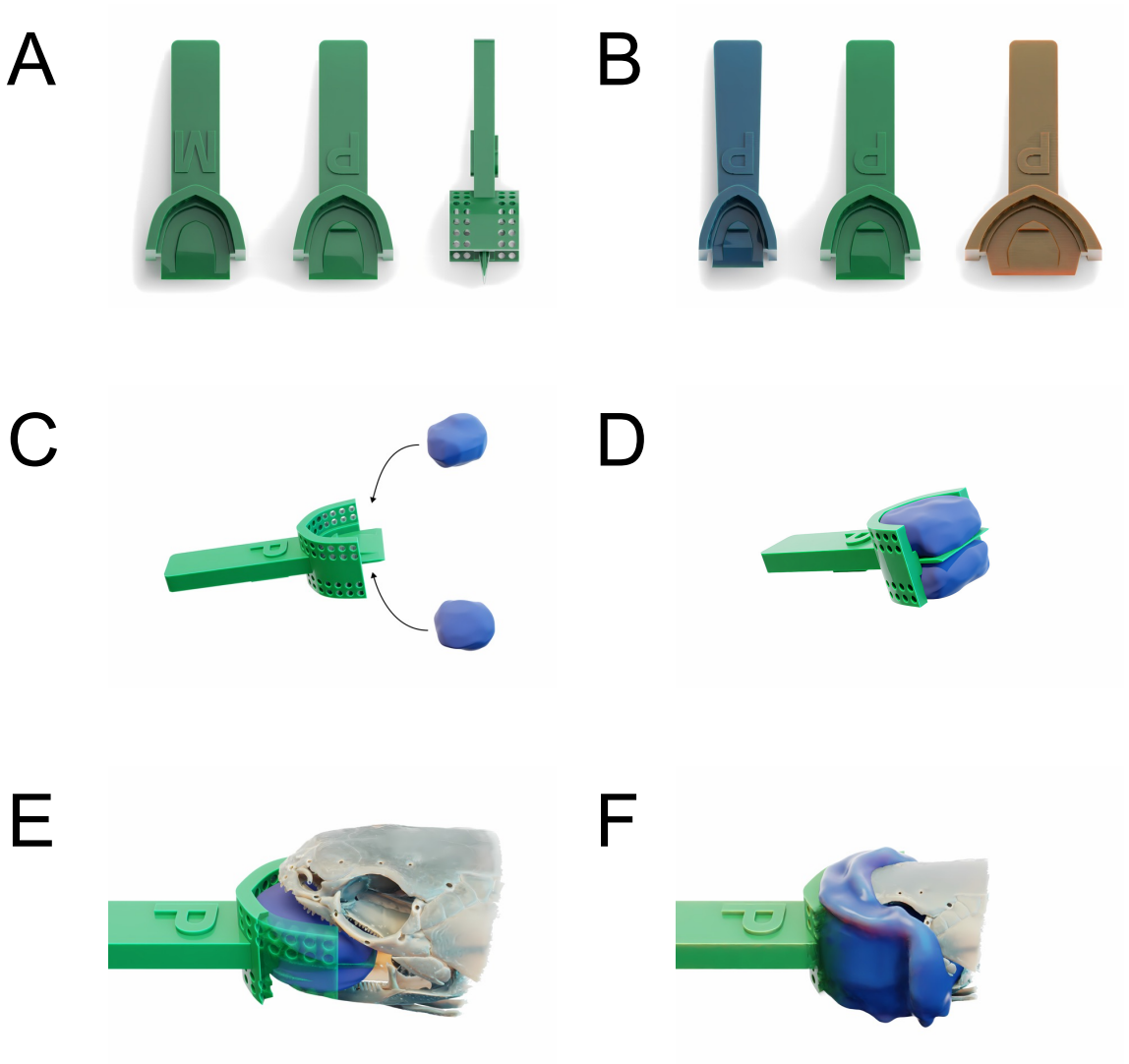
Schematic representation of the dental impression tray used to mold *Polypterus* teeth and the following process. (A) 3D rendered image of the impression tray of mandibular side, palatal side, and side view. (B) Impression trays in multiple different sizes. (C-D) Impression material is applied to both sides of the impression tray. (E-F) *Polypterus* biting on the impression material.

Figure 2. Molding sequence

Molding sequence of *Polypterus*. (A) Preparation of materials before starting the procedure (B) Individual in buffered MS-222 bath. (C) Removal of residual moisture using compressed air duster. (D-E) Removal of impurities from teeth surface using putty. (F) Application of PVS material on the surface of the impression tray. (G-H) Molding of the teeth by firmly pushing the jaw onto the impression tray.

Figure 3. Obtained impressions.

PVS mold obtained upon the completion of molding. (A-B) Undetached mold taken upon molding from (A) palatal side and (B) mandibular side. (C-E) Lingual scans of mold obtained from the palatal side for (C) posterior tooth, (D) anterior tooth, and (E) the mandibular side for posterior tooth. (F-G) Lingual scan of the mandibular side for a middle tooth, which enamel was flaked off by brushing, (F) photo-simulation and (G) its topology showing the elevation at the area where enamel was flaked off. The size of photo-simulations (C-F) is 100 micrometers (Y axis) times 140.5 micrometers (X axis).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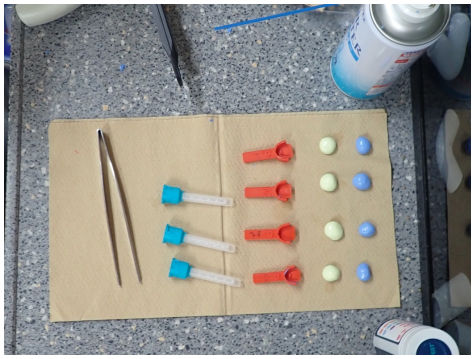

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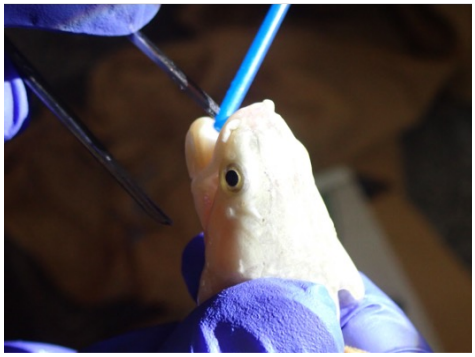

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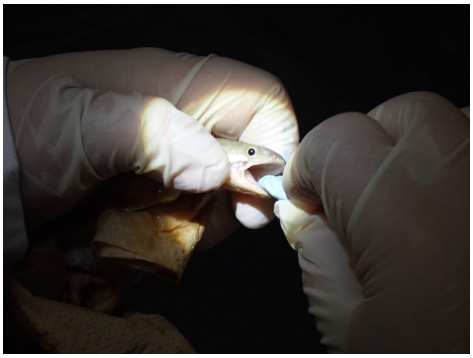

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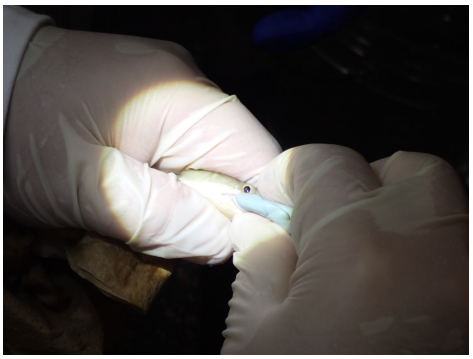

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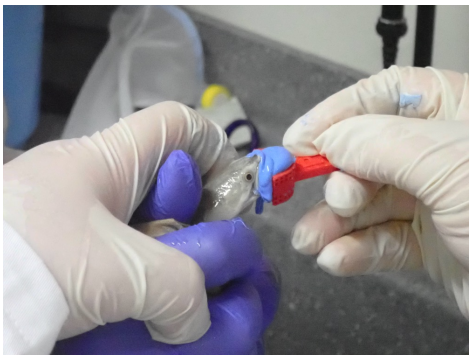

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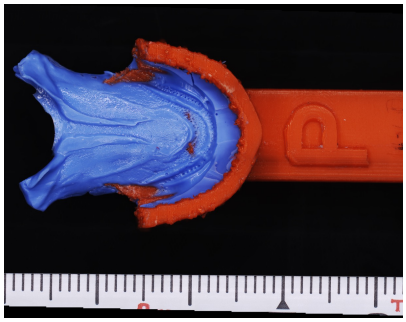

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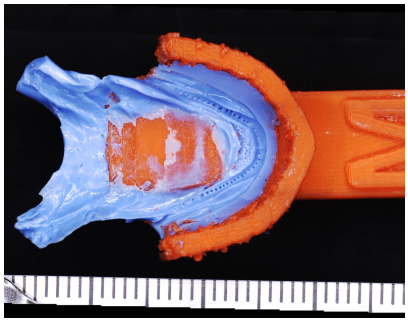

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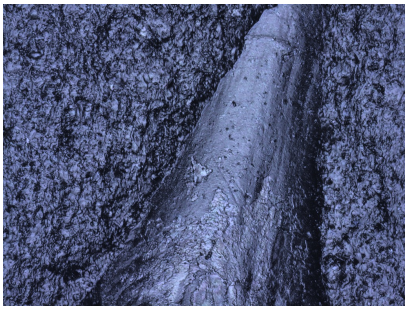

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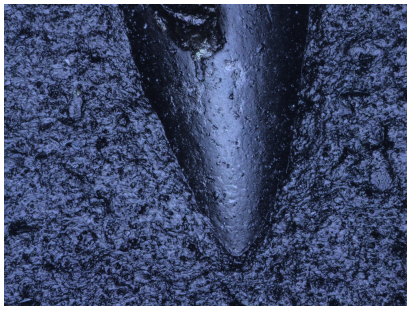

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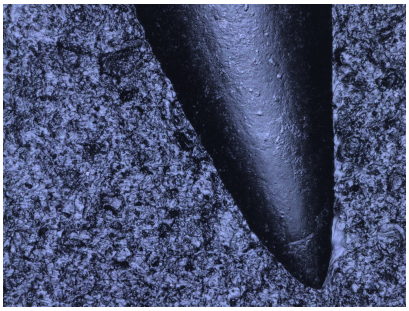

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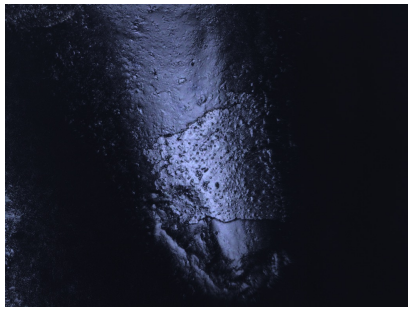

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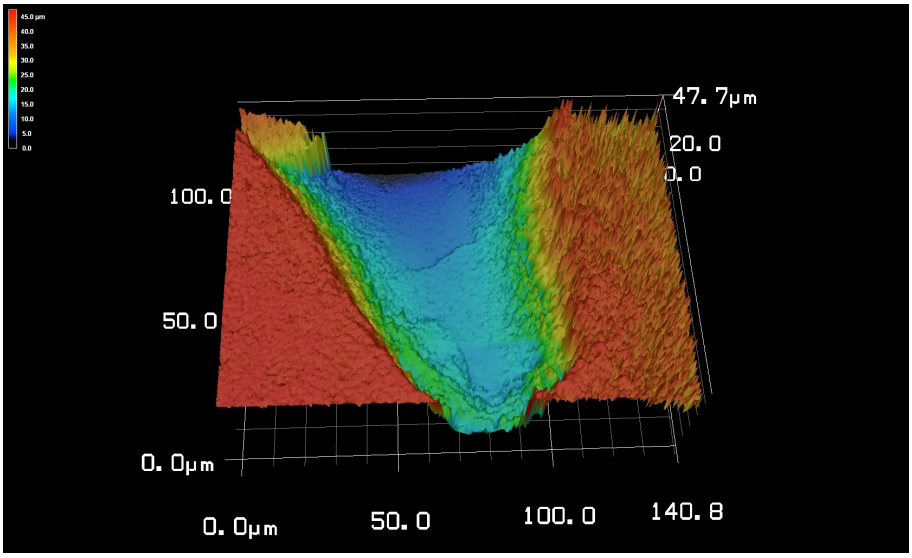
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