SHORT TERM CONTRACT FOR THE BIOLOGICAL STUDIES (ICCAT GBYP 05/2021) OF THE ATLANTIC-WIDE RESEARCH PROGRAMME FOR BLUEFIN TUNA (GBYP Phase 11)

APPENDIX 1

Assessment of the potential of epigenetic approaches for ageing Atlantic Bluefin tuna samples

Naiara Rodríguez-Ezpeleta (nrodriguez@azti.es)

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1. Context

Previous work has suggested that methylation profiles could be used to assign age in commercial fish species. Yet, obtained age estimations have quite high error margins (compared to otolith derived ages), which could prevent the implementation of this method for Atlantic Bluefin tuna assessment, including future close-kin-mark recapture endeavours. Here, we evaluate the potential of epigenetic approaches for ageing Atlantic Bluefin tuna individuals. For that aim, we have performed a bibliographic search to compile all studies using epigenetics for aging fish from which a viability study will be derived including required logistics, costs, and anticipated error margins. The obtained information has been contrasted with the data required for assessment and close-kin mark recapture to evaluate the potential of this approach and its advantages and disadvantages over existing methods.

2. Epigenetics and ageing

Epigenetics can be defined as the ensemble of processes that alter gene activity without changing the DNA sequence and which can be heritable and also reversible (Weinhold 2006). Thus, epigenetic changes are those changes in the genome that do not involve changes in the nucleotide sequences, but which affect gene expression. Several types of epigenetic processes have been described, the most common being histone modifications, RNA-based mechanisms, and DNA methylation, (Figure 1). Among epigenetic mechanisms, DNA methylation, which consists of the addition of methyl groups to the DNA, typically cytosines within cytosine-phosphate-guanine (CpG) sites, has been considered as a potential predictor of age (Horvath 2013), since there has been shown that there is a relationship between methylation changes and age (Field et al. 2018).

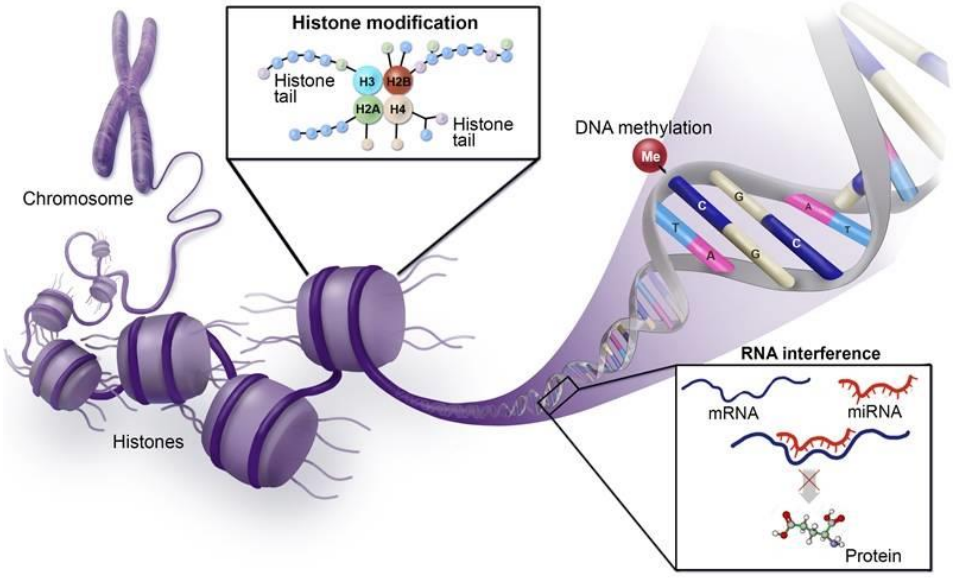

Figure 1. Graphical representation of the main epigenetic mechanisms (Source: https://www.hematology.org/research/ash-agenda-for-hematology-research/epigenetic-mechanisms)

This has lead to the development of the so-called epigenetic clocks in humans, mice, dogs, wolves and humpback whales, among others (Field et al. 2018). The clock is developed using a linear regression algorithm that trains against the chronological age of the samples and selects a set of age-informative CpG dinucleotides, weighed by their contribution to the clock according to the magnitude of its age-dependent change (Figure 2). Then, the accuracy of the clocks is measured by the correlation coefficient between actual chronological age and measured age and by the average absolute difference between actual age and measured age. Two of the most accurate human clocks have correlation coefficients > 0.9 and average errors of less than 5 years (Hannum et al. 2013; Horvath 2013).

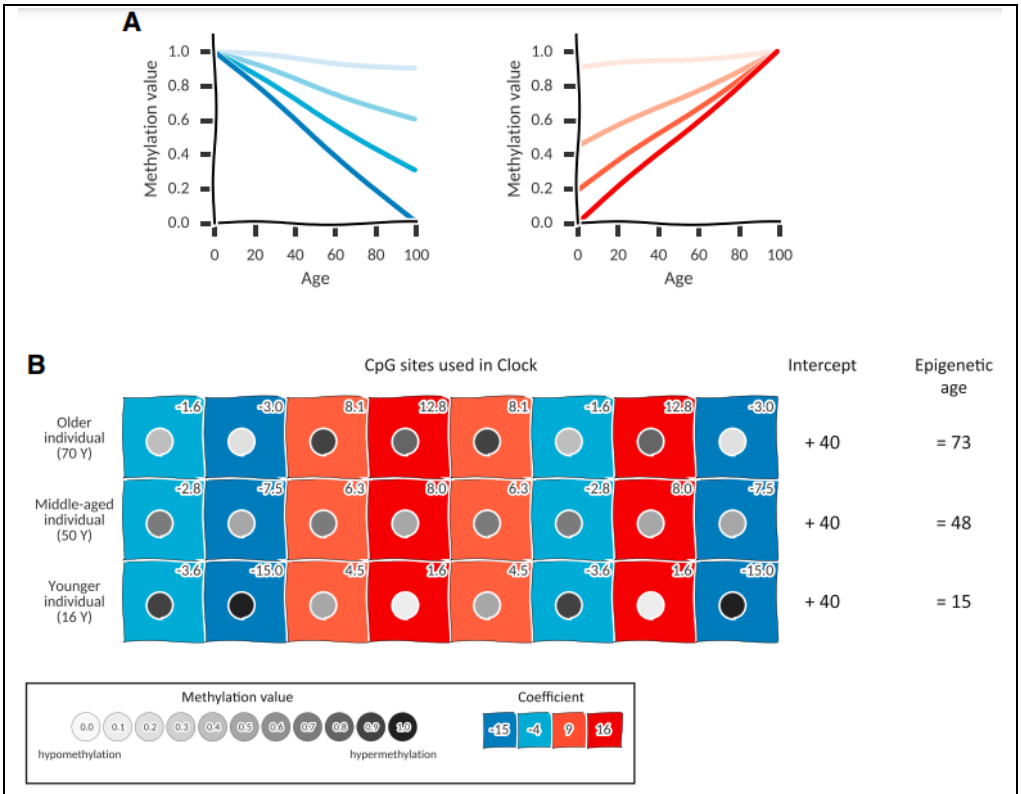

Figure 2. Illustrative example of (A) CpG methylation level changes with age at four negative (blue) and four positive (red) coefficient sites and (B) calculation of epigenetic age in three individuals using methylation values and coefficient changes. Figure from Field et al. (2018)

3. Epigenetic clocks in fish

Previous studies on zebrafish, chinook salmon and steelhead trout showed potential for the development of an epigenetic clock in fish (Shimoda et al. 2014; Venney, Johansson, Heath 2016; Gavery et al. 2019). However, because the content of methylated cytosines (^{5m}C) is inversely related to body temperature, fish, being cold-blooded, have a higher number of ^{5m}C than warm-blooded vertebrates such as mammals and birds. Also because ^{5m}C content is dependent on temperature, fish living in polar and tropical regions will have high and low ^{5m}C content respectively (Varriale 2014). Also, compared to mammals and birds, fish have an indeterminate growth and age increased fecundity. All this points to uncertainties regarding the development of an epigenetic clock in fish. Yet, recent examples (Table 1) on the development of epigenetic clocks in fish show promise.

Table 1: Summary of epigenetic clocks in fish (available as excel file)

Species	N	Environment	Tissue	Method	Number of CpG	Compared with	Lifespan	Age range	Accuracy (correlation)	Precision (MAE)	Reference
Seabass (*Dicentrarchus labrax*)	88	Captive	muscle, testis, ovaries	Bisulfite treatment & amplification of 4 candidate genes selected based on previous knowledge	48 initial; 28 correlated with age	Known age	25 years	0.55-10.5 years	0.824	2.15 years	Anastasiadi & Piferrer. MER (2019)
Zebrafish (*Danio rerio*)	86	Captive	Fin	Reduced representation bisulphite sequencing (RRBS)	1,311 correlated with age, 29 selected and 26 translated into multiplex PCR	Known age	182 weeks	10-78 weeks	0.92	3.7 weeks	Mayne et al. Aging (2020)
Australian lungfish (*Neoceratodus forsteri*)	141	Wild	Fin	Bisulfite treatment & gene specific amplification; primers desigend based on RNA-seq data	31 age associated sites from the 1,311 identified in zebrafish were captured by alginment	Bomb radiocarbon dating and known age	85 years	1-77 years	0.98	0.86 years	Mayne et al. MER (2021)
Murray cod (*Maccullochella peelii*) Mary River cod (*M. mariensis*)	33/37	Wild/wild and captive	Fin	Bisulfite treatment & gene specific amplification; primers desigend bsaed on genome	26 age associated sites from the 1,311 identified in zebrafish were captured by alginment	Otolith ring count dating/know n age	48 years	1-12 years	0.92	0.34 years	Mayne et al. MER (2021)
Red snapper (Lutjanus campechanus)	10	Wild	Muscle	Bisulfite-converted restriction site associated DNA sequencing	3224 correlated with age; 199 selected	Otolith ring count	50 years	1-26 years	1	N/A	Weber et al. CJFAS (2021)
Red grouper (*Epinephelus morio*)	10	Wild	Fin	Bisulfite-converted restriction site associated DNA sequencing	690 correlated with age; 49 selected	Otolith ring count	25 years	2-14 years	1	N/A	Weber et al. CJFAS (2021)
Southern bluefin tuna (*Thunnys maccoyii*)	125	Wild	Fin	Most likely done as above	? number of sites discovered from the 1,311 identified in zebrafish were captured by alginment	Otolith ring count	40 years	1-38 years	0.92	1.7 years	Mayne et al. Repport to CCSBT (2021)

Seabass

The first epigenetic clock developed in fish was for seabass (*Dicentrarchus labrax*) (Anastasiadi, Piferrer 2020). For that aim, they used 88 individuals (age range of 0.55-10-5 years) in captivity of known age and selected 4 genes based on the absence of sex-related differences in DNA methylation and major gene expression changes. The epigenetic clock was constructed using four candidate genes selected based on the absence of sex-related differences in DNA methylation and major gene expression changes. Initially 48 CpGs were selected from which 24 showed correlation with age. The clock had a correlation of 0.824 and a mean absolute error of 2.15 years (Figure 3). The clock developed on muscle tissue in cold water reared fish worked well in fish reared at low and high temperature (tested only in young fish; <2 years) and worked well in testis but not in ovaries. Seabass can live up to 25 years and the oldest individuals used in this study were 10.5 years; thus, the model was not developed nor tested in older individuals.

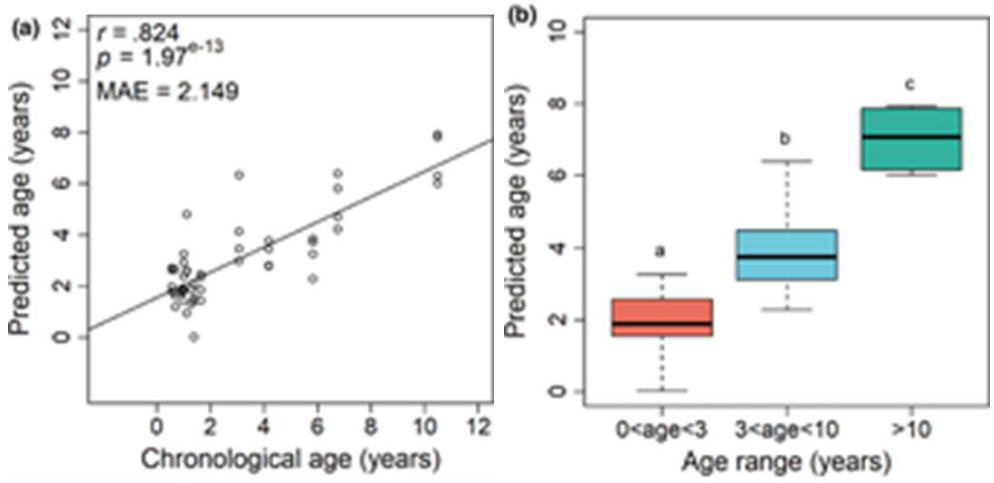

Figure 3. Performance of the model based on 48 CpG sites and boxplots showing absolute error rates in each age range. Figure from Anastasiadi, Piferrer (2020)

Zebrafish

For developing an epigenetic clock in zebrafish (*Danio rerio*) (Mayne et al. 2020) a "blind" approach based on assessing the methylation levels of a large fraction of the genome was used, the so called, reduced representation bisulfite sequencing (RRBS). They discovered more than half a million CpG sites, from which 1,311 had methylation changes that were correlated with age. They used 86 individuals in captivity of known age of which 70% were included in the training set and 30% in the test set. The minimum number of sites required to estimate age was 29. The correlation in the test set was of 0.92 and the mean absolute error was of 3.7 weeks (Figure 4). In order to facilitate further analyses, they developed a multiplex PCR assay for the 29 identified CpG sites, of which 3 failed. Zebrafish can live up to 182 weeks and the oldest individuals used in this study were 78 weeks; thus, the model was not developed nor tested in older individuals. Additionally, the model was developed for a particular strain and for individuals in captivity, which implies that it needs to be tested on other strains and no wild caught fish.

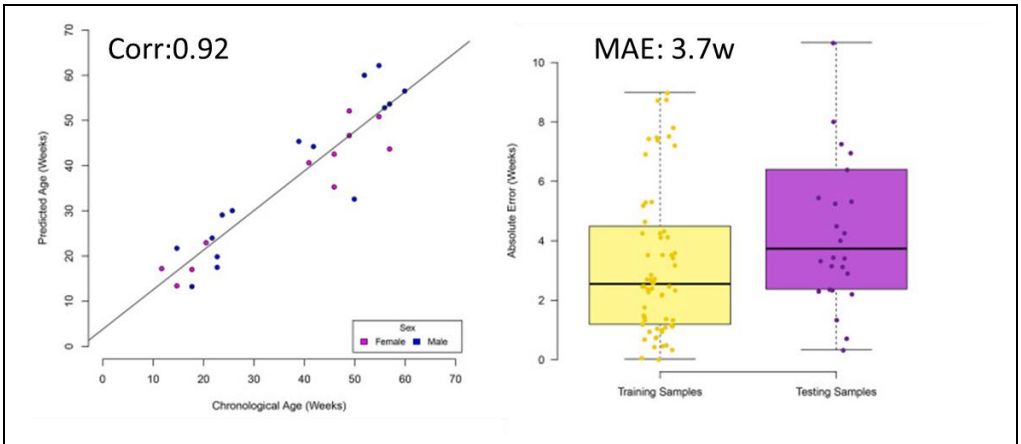

Figure 4. Performance of the model based on 29 CpG sites in the test dataset and boxplots showing absolute error rates in training and test datasets. Figure from Mayne et al. (2020)

Australian lungfish

The development of the epigenetic clock in the Australian lungfish (*Neoceratodus forsteri*) (Mayne et al. 2021a) was based on the CpG sites identified for zebrafish (Mayne et al. 2020). Wild caught samples of known age (39) and bomb radiocarbon dated (102) were used and 31 CpG sites could be identified by aligning RNA-seq data of the target species to the zebrafish genome. The correlation was of 0.98 and the mean absolute error was of 0.86 years (Figure 5). Although the mean absolute error is apparently small, this increases in older specimens, being of more than 6 years in individuals older than 40 years.

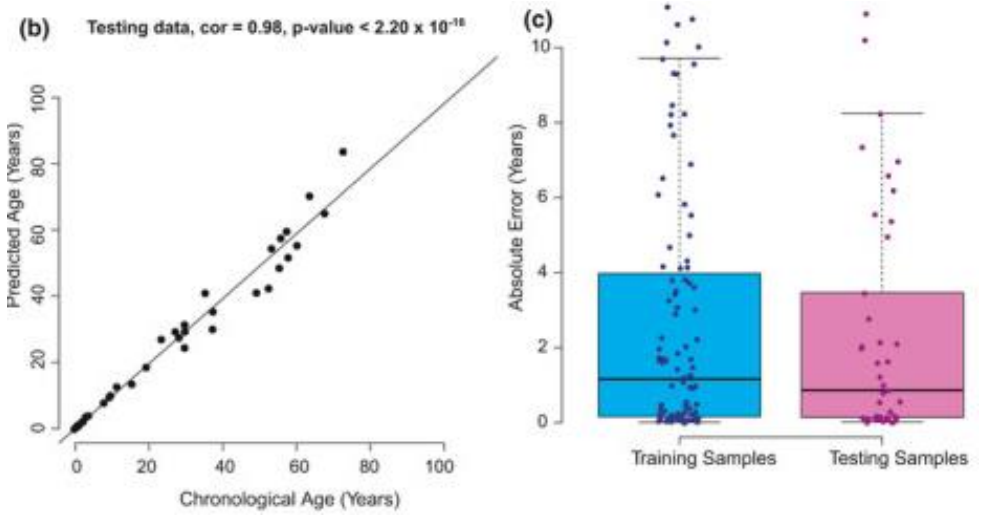

Figure 5. Performance of the model based on 31 CpG sites in the test dataset and boxplots showing absolute error rates in training and test datasets. Figure from (Mayne et al. 2021a)

Murray and Mary River cod

The development of the epigenetic clock in the Murray and Mary River cod species (*Maccullochella peelii* and *Maccullochella mariensis*) (Mayne et al. 2021a) was based on the CpG sites identified for zebrafish (Mayne et al. 2020). 33 wild caught otolith ring count dated samples and 37 wild caught known age samples were used respectively for each species and 26 CpG sites could be identified by aligning genome data of the target species to the zebrafish genome. The correlation was of 0.92 and the mean absolute error was of 0.34 years (Figure 6).

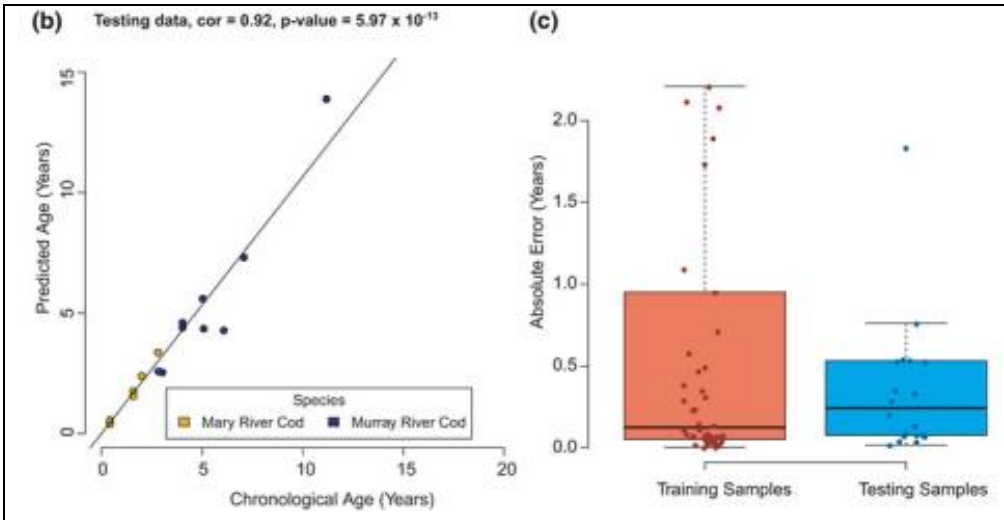

Figure 6. Performance of the model based on 26 CpG sites in the test dataset and boxplots showing absolute error rates in training and test datasets. Figure from (Mayne et al. 2021a)

Red snapper and red grouper

The development of the epigenetic clock in the red snapper (*Lutjanus campechanus*) and red grouper (*Epinephelus morio*) was based on a "blind" approach based on assessing the methylation levels of a large fraction of the genome (Weber et al. 2021). This approach is similar to the one used in zebrafish but covering less sites while being also less costly (Trucchi et al. 2016). 10 otolith ring count aged specimens were used per species. In red snapper, from almost 50,000 sites identified, 3224 exhibited age-correlated methylation and 199 were selected; in red grouper, from more 9000 sites recovered, 690 exhibited age-correlation methylation and 49 were selected. The correlation between the estimated age and the chronological age was of 1, which is most likely due to the fact that the same samples used for CpG site selection were used for correlation assessment.

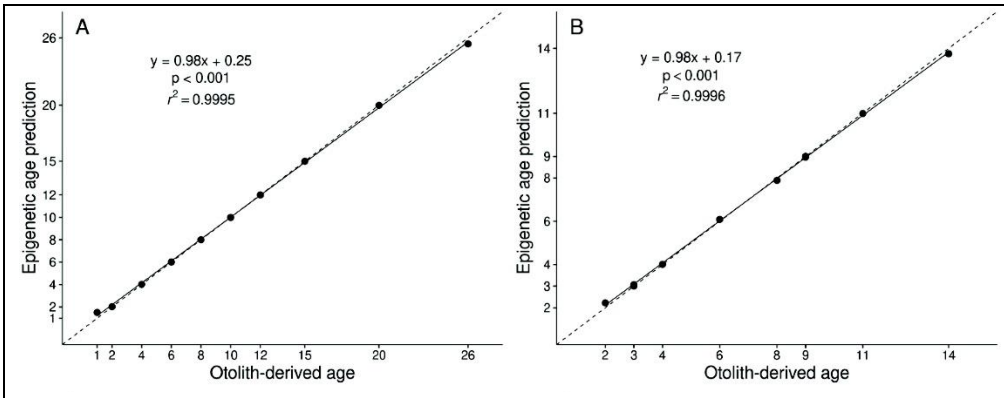

Figure 7. Performance of the model for red snapper and red grouper. Figure from (Weber et al. 2021)

Southern Bluefin Tuna

The development of the epigenetic clock in the Southern Bluefin tuna (*Thunnus maccoyii*) (Mayne et al. 2021b) is ongoing and the available data for the moment is contained in an CCSBT report; no publications are available yet and therefore, there is a lack of information on used methodology and identified CpG sites. The method was most likely based on the CpG sites identified for zebrafish (Mayne et al. 2020). 125 wild caught otolith ring count dated samples were use and an unknown number of CpG sites could be identified. The correlation was of 0.92 and the mean absolute error was of 0.34 years (Figure 8).

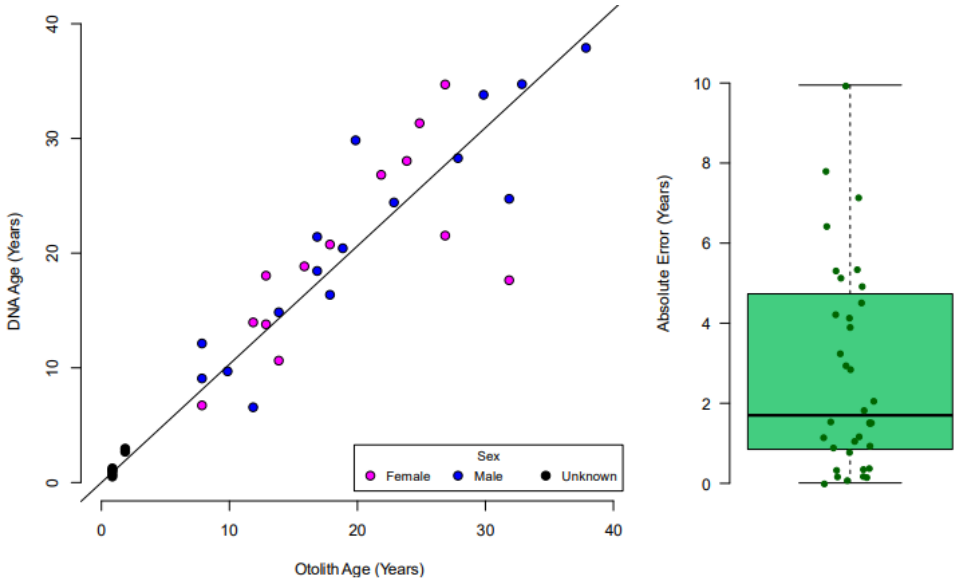

Figure 8. Performance of the model based on an unknown number of CpG sites in the test dataset and boxplots showing absolute error rates in training and test datasets. Figure from (Mayne et al. 2021b)

4. Considerations for an epigenetic clock on Atlantic Bluefin tuna

4.1. Samples for the training and test sets

Considering variables such as environment (2), sex (2), genetic components (3), and age classes (8) and including 3 replicates for each combination, a first proposal could include about 288 samples of which 192 should be used for training and 96 for testing (Figure 9). Se details below.

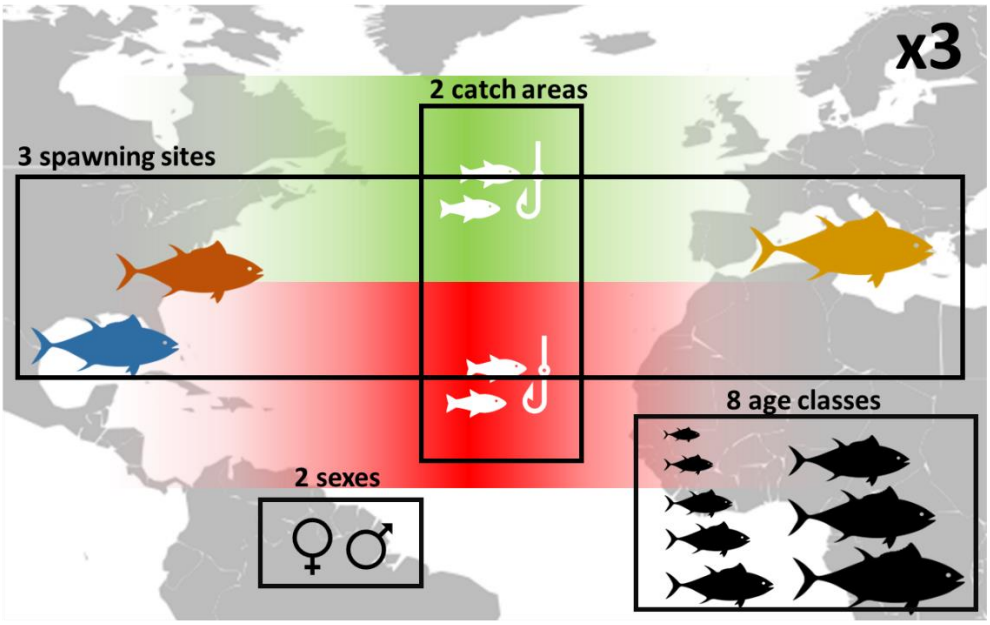

Figure 9. Sample set proposal for a prospective epigenetic-clock study in Atlantic Bluefin tuna. Three spawning sites (aka. Genetic components) are considered; ideally feeding aggregates (of known spawning component) caught at northern and southern latitudes should be included as well as individuals from both sexes and at least 8 age classes.

Environment

Because methylation profiles not only depend on age but on other factors such as sex, environment, or diet (REF), a study design aimed at identifying age associated methylation should minimize influence of confounders and false positives; this can be done by reducing all variables except age, which is only possible in captivity reared individuals, or by including as many variables as possible. Previous studies have followed the former strategy (zebrafish and seabass studies were focused on captive individuals) or have focused on species with very localized distribution where environmental effects are assumed limited (the Australian lungfish and the Murray and Mary River cod inhabit a few rivers in Australia). Two cases have included wild caught individuals. The red snapper and red grouper study included only individuals from the Gulf of Mexico, whereas the species have a much wider distribution and meaning that the samples used are not representative of the species. The Southern bluefin tuna inhabits a quite narrow latitudinal range (30°S –50°S) and has a unique spawning site in the northeastern Indian Ocean south of Java suggesting that the samples used could be a good representation of the species.

The Atlantic bluefin tuna has at least three known spawning sites (Mediterranean Sea, Gulf of Mexico and Slope Sea) and has a wide distribution range (20°N – 60°N). Also, they are known to be able to thermoregulate their body temperature and live in various seawater temperatures. This implies that **samples used for an epigenetic clock in Atlantic bluefin tuna should include individuals born at the three spawning sites and, as far as possible (although difficult to have the information), individuals that have inhabited southernmost and northernmost latitudes.**

Sex:

Because methylation profiles associated to age could differ between sexes and genetic profiles, samples used for training should include samples from both sexes. The seabass and zebrafish studies used individuals of both sexes in their test and training sets, whereas no information on sex was provided for the red grouper and red snapper, the Australian lungfish, nor the Murray and Mary River cod. They did use individuals of both sexes in the Southern bluefin tuna study.

Due to potential differences for sex, **samples used for an epigenetic clock in Atlantic bluefin tuna should include individuals of both sexes**, males and females at least for the adult individuals. For younger individuals, a genetic test for sex could be applied if no reproductive organs can be examined.

Genetic component:

Because methylation profiles could also be associated to genetic component or population, samples used for training should include samples representing the whole genetic variability of the specie. The seabass and zebrafish studies included captive individuals, which implies that only one genetic component was assessed and that it is not clear that the clock will perform well in other populations or strains (Mayne et al. 2020). In the cases of the Australian lungfish, Murray and Mary River cod and of Southern bluefin tuna only one genetic component is assumed.

Due to potential differences for genetic components, **samples used for an epigenetic clock in Atlantic bluefin tuna should include individuals the three recognized genetic components**. This will be achieved by collecting samples born or spawning at the three main spawning grounds, but ideally their genetic makeup should be examined to assess potential differences in methylation profiles.

Age classes:

To have a good model for the epigenetic clocks, ideally all age classes should be used. In previous studies, some species have not included the oldest specimens (e.g. seabass, zebrafish and Murray and Mary river cod), making it impossible to assess the performance of the model in advanced age classes. Only the Australian lungfish and the Southern bluefin tuna cases have included oldest individuals, resulting in higher error rate in oldest age classes (see section on error rates below).

Due to the need of having a model that performs well at all age classes, **samples used for an epigenetic clock in Atlantic bluefin tuna should include individuals spanning the age distribution of Atlantic Bluefin tuna**, that not only youngest individuals (>1year), but also oldest (40 years). The age ranges to be included should be defined depending on needs for CKMR; at least 8 should be included (plus group?). These individuals should have as accurate as possible

age estimates, which is the case of Atlantic Bluefin tuna will be achieved by otolith ring reading. Possibility of using captive individuals from Baleares?

4.2. Identification and Selection of CpG sites

The number of CpG sites required for epigenetic-clock development depend on many factors such as tissue type, assay design, biological and technical variability. Ideally, a reduced set of age-informative CpGs should be identified for each species/group of species if universal epigenetic-clocks are envisaged. Once these sites identified, specific assays to type them which could be quite cost-effective (5-10€) can be developed. But first, the sites need to be selected, which requires some initial investment. Although the final set of age-diagnostic CpGs could be of tens or hundreds, the largest the number of CpGs analyzed to select the final set, the more precision the clock will have. For example, studies in mouse started from about 1 million CpG sites to select a final set of 329 (Stubbs et al. 2017), studies in wolves and dogs started from about 200K CpGs to select a final set of 115 (Thompson et al. 2017). Several alternatives for discovering age-informative CpG sites are possible, going from candidate gene selection, transfer of previously identified CpGs from other species or identifying new CpGs based on a reduced fraction or on the whole genome. The epigenetic-clock studies on fish performed to date have used different approaches for selecting age-informative CpG sites.

Candidate gene approach:

The candidate gene approach was used in the seabass study (Anastasiadi, Piferrer 2020). This is a risky approach because only a few genes (selected based on other criteria than being informative for age) were used, from which 48 CpGs were identified. This is a very low starting number, and therefore it is quite surprising that a final set of 28 age-correlated CpG sites could be derived from them. Yet, in this case it seems that both the model and the CpG sites was selected on the same samples used for accuracy and precision calculation, which could explain the very good performance of the clock.

Due to the lack of candidate genes for an epigenetic-clonk in Atlantic bluefin tuna, **the candidate gene approach can be discarded for an the development of an epigenetic clock in Atlantic bluefin tuna.**

Transfer from other species:

An alternative approach for the identification of age informative markers is to transfer them among taxa. In humpback whales (*Megaptera novaeangliae*) and Bechstein's bats (*Myotis bechsteinii*), age estimation models were developed using conserved age- associated CpG sites in humans and mice (*Mus musculus*) (Polanowski et al. 2014; Wright et al. 2018). Also, in a recent study, it has been found that CpG sites across 128 mammalian species can be predictive of age (Mammalian Methylation et al. 2021). This approach was used in the Australian lungfish, the Murray and Marry River cods (Mayne et al. 2021a) and the Southern

bluefin tuna (Mayne et al. 2021b). In the two first cases 31 and 26 CpG sites were found to correlate with age from the 1,311 found in zebrafish (no information is yet available for the Southern Bluefin tuna case).

The fact that no common sites could be found between the Australian lungfish and river cods could question the universality of the clocks in fish and it is yet to be tested if the 1,311 markers form zebrafish could be used as the base from which age-informative CpG sites for other species could be detected. Also, because the Atlantic Bluefin tuna has a so wide range distribution and genetic components, ideally, the development of an epigenetic clock should be based on a large initial set of CpG sites. Thus, we consider that **the transfer to other species approach could be a risky approach for the development of an epigenetic-clock in Atlantic bluefin tuna.**

Reduced representation sequencing:

As opposed to the other two approaches, the reduced representation approach does not require any candidate genes or information from other species and can be used to identify CpG sites "de novo". This is the approach that was used in the zebrafish (Mayne et al. 2020) and the red grouper and red snapper (Weber et al. 2021), where an initial set of tens to hundreds of thousands CpG sites were identified from which a small percentage (0,25-1%) were considered age-informative. This reduced percentage of age informative CpGs found questions the candidate gene approach and potentially also the transfer from other species approach. For this reason, we believe that **the reduced representation sequencing approach could be a reasonable alternative for the development of an epigenetic-clock in the Atlantic bluefin tuna.** This method would also require a high-quality whole genome sequence of Atlantic bluefin tuna. So far, there is one version available (https://www.ncbi.nlm.nih.gov/data-hub/taxonomy/8237/), which could be enough.

Whole genome sequencing:

Another alternative would be to screen all the CpG sites in the genome and not only a reduced fraction. This approach has not yet been applied for developing epigenetic-clocks in fish, but the continuously decrease in price of sequencing is making it now more affordable and almost at the same cost as the reduced representation sequencing. We believe that the genome requirements would be the same as for the reduced representation sequencing approach, but we are in the process of inquiring this with the sequencing company.

4.3. Error rates

Obtained age estimations have variable error margin, which in general increase in older specimens. For example, in the seabass study, the mean error rate is of 2.15 years; analysing this error rate per sample we have extreme cases were a

3-year-old fish is estimated to be 6.5 years old, a 5.8 year old fish is estimated to be 2 years old and a 10.5 year-old fish is estimated to be 6 years old (Table 2).

Table 2: Minimum and maximum epigenetic-clock predicted age for the 5 oldest age classes in seabass

Chronological age	Predicted age (min)	Predicted age (max)
3.07	3	6.5
4.17	2.75	4
5.83	2	5
6.75	4	6.5
10.5	6	8

In the case of the Australian lungfish the mean error rate is of 0.86 years, apparently very small, but there is an error of > 10 in oldest specimens. Perhaps this could be considered acceptable considering that the alternative is no information. In the case of the southern bluefin tuna, the mean error rate is of 1.7 years, but for some individuals the error is of more than 10 years. Thus, although there is a correlation between epigenetic and chronological age, the differences between the two could be high. Also, in the southern bluefin tuna, the chronological age of the specimens is estimated using otolith ring counts, which means that the chronological age could not be well estimated. In the case of red snapper and red grouper, the error rate is almost zero which could be explained by the fact that the samples used for clock development were used for clock testing.

What is the error rate we can afford for an epigenetic clock to be useful? This depends mainly on:

- if epigenetic clock is the only alternative, that is if otolith reading cannot be used because not accurate and/or destructive and age-length-keys provide higher error rates than epigenetic-clocks
- if epigenetic clock is cheaper than alternatives that provide more accurate, but not perfect age estimations
- if the error rates implicit in the epigenetic clock do not affect the application of the CKMR (e.g. accurate age is not possible, but binning samples into age classes is)

4.4. Costs

The costs implicit in an epigenetic clock development for the Atlantic bluefin tuna should consider sampling, CpG site identification and selection, species-specific assay development and developed epigenetic clock testing in alternative

samples. Once the assay developed, it is estimated that the cost per sample will be of about 5-10€.

The costs of developing the assay are variable and depend on the approach used and on the success of the different steps involved in the process. The use of a candidate gene approach is the cheapest alternative but could be risky as it is possible that no markers are found or that the ones found are not discriminative enough and imply large error rates. Alternatively, the reduced representation sequencing and whole genome analyses approaches imply a cost of 100-250€ per sample included in the development process. One possibility would be to include 96 samples as training to which these approaches could be applied and use the 192 others as test set once the assay is developed.

5. Conclusions

- The development of an epigenetic clock in Atlantic bluefin tuna requires a **samples that ensure good representation of the species population** in terms of environment, genetic component, sex and age classes
- The samples used in the development and testing of the method will be **aged using otolith ring count analyses**, which could already bias the results, if this measurement is not considered accurate
- The method for CpG site identification should **ensure that the best set of informative markers is found** and for that aim, the reduced representation or whole genome sequencing are the best approaches.
- **The error rates from previous studies are high for the oldest specimens; using a large set of training samples, a good chronological ageing method and a large set of CpG sites will reduce this error.**
- We should evaluate if the error rates expected (based on previous studies on long-lived species) are compatible with the application of the CKMR and **if the reduced cost and logistics implied in epigenetic clock ageing compensate the implicit error rates**

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