Automated staging of zebrafish embryos with KimmelNet

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Abstract

The zebrafish (*Danio rerio*), is an important biomedical model organism used in many disciplines. The phenomenon of developmental delay in zebrafish embryos has been widely reported as part of a mutant or treatment-induced phenotype. However, the detection and quantification of these delays is often achieved through manual observation with reference to staging guides, which is both time-consuming and subjective. We recently reported a machine learning-based classifier, capable of quantifying the developmental delay between two populations of zebrafish embryos. Here, we build on that work by introducing a deep learning-based model (KimmelNet) that has been trained to predict the age (hours post fertilisation) of individual zebrafish embryos. We show that when KimmelNet is tested on 2D brightfield images of zebrafish embryos, the predictions generated agree closely with those expected from the Kimmel equation. Random sampling of the test data demonstrate that KimmelNet can be used to detect developmental delay between two populations with high confidence based on as few as 20 images of each population. Finally, we show that KimmelNet's predictions are resilient against low levels of additive noise. With the ability to analyse tens of thousands of standard brightfield microscopy images on a timescale of minutes, we envisage that KimmelNet will be a valuable resource for the developmental biology community. Furthermore, the approach we have used could easily be adapted to generate models for other organisms.

Introduction

The zebrafish (*Danio rerio*) is a commonly-used model organism in the field of developmental biology, with their transparent embryos making them particularly conducive to microscopy observation. Many developmental studies require accurate staging of zebrafish embryos and larvae. Although the timing of fertilisation can be estimated to within approximately 30 minutes, the hours post fertilisation (hpf) at the standard temperature of 28.5°C provides only an approximation of the actual developmental stage, because other factors, like population density and water quality, can affect maturation (Singleman and Holtzman, 2014). Even controlling for such factors, embryos within a clutch may develop at different rates (Parichy et al., 2009). Researchers therefore use both hpf and staging guides that are based on morphological criteria to stage individual embryos. These morphological features include the number of somites and the appearance of landmark structures such as the embryonic shield, tail bud and eye primordium (Kimmel et al., 1995; Westerfield, 2000).

Staging of embryos is of particular importance because many studies report 'developmental delay' as part of a genetic or drug-induced phenotype (Akthar et al., 2019; Byrnes et al., 2018; Elabd et al., 2019; Farooq et al., 2019; Giraldez et al., 2005; Jia et al., 2020; Li et al., 2017). Zebrafish have also emerged as important models in which to study the effects of environmental toxins, with many of these treatments also resulting in developmental delay (Aksakal and Sisman, 2020; Li et al., 2020; Mesquita et al., 2017). Such delays are difficult to quantify without manually staging large numbers of embryos, which is subjective and time-consuming (Jeanray et al., 2015; Teixidó et al., 2018). A more standardized and automated approach to assessing developmental delay is therefore desirable, in order to avoid the substantial time cost and subjectivity associated with manual staging.

The use of image analysis has become increasingly popular in the life sciences in recent

years, facilitating the automated quantification of microscopy images in an unbiased fashion (Meijering et al., 2016). But, designing an image analysis algorithm to specifically detect the wide range of morphological features on which staging guides depend would be a challenging endeavour. However, the staging of embryos based on microscopy images could also be considered an image classification task, something to which machine learning is well-suited. Machine learning approaches, where a computer program uses algorithms and statistical models to continuously learn and improve pattern prediction, is already used widely in biological studies (Greener et al., 2022).

Machine learning has found some use in the analysis of zebrafish embryos, but mainly for phenotype classification, rather than assessing developmental delay. For example, using a support vector machine, Liu et al. (2012) achieved a classification accuracy of approximately 97% in differentiating between hatched, unhatched and dead zebrafish embryos. Jeanray et al. (2015) used a supervised machine learning approach to place brightfield images of 3 day old zebrafish larvae in one of eleven phentotypic classes. But their method involved significant image pre-processing and their classifier failed to accurately classify certain phenotypes.

More recently, convolutional neural networks (CNNs), have grown in popularity in life science research in general, and they are particularly well-suited to the analysis of image data (Greener et al., 2022). There are a number of examples in the literature of CNNs being used for zebfrafish embryo phenotype classification. Ishaq et al. (2017) used a CNN-based approach to predict the probability of deformation in multi-fish microwell plates. While few images were needed for training (< 100) and an F score of 0.915 was achieved, their network could only differentiate between two different classes. Tyagi et al. (2018) tested multiple CNN architectures to classify zebrafish embryo phenotypes, but achieved a relatively low accuracy (83.55%) when attempting to classify a large number of phenotypes (11). Using the image data from Jeanray et al. (2015), Shang et al. (2020) achieved a higher

accuracy using a two-tier CNN-based approach, but again, the number of images used for training (529) and testing (341) across ten classes was limited.

In terms of developmental staging, Pond et al. (2021) developed a convolutional neural network (CNN) to stage zebrafish tail-buds at four discrete developmental stages just 1.5 hours apart. Although they trained their network with a small number of images (< 100) and achieved 100% test accuracy in some cases, the number of test images per class was in several cases as low as 2.

We recently published a machine-learning based method for the staging of zebrafish embryos (Jones et al., 2022). While this method successfully discriminated between different populations of zebrafish developing at different rates, the developmental profiles it produced were distinctly non-linear. This was largely due to the fact that the underlying classifier was trained to recognise two distinct classes corresponding to two different stages of development (embryos at 4.5hpf and 17.5hpf). As such, it performed poorly at accurately staging embryos in between those specific time-points, producing a developmental profile with a distinctive 'S' shape.

Here we present a more robust classifier, based on a convolutional neural network, that provides more accurate predictions of developmental stage across a much wider range of timepoints (4.5 to 51 hpf). Named KimmelNet and using simple brightfield microscopy images as training data (with no manual annotation required), the developmental profiles produced by our CNN closely follow those predicted by the Kimmel equation (Kimmel et al., 1995). We demonstrate that KimmelNet accurately quantifies developmental delay between two populations of zebrafish embryos. We also demonstrate that KimmelNet's performance is not degraded by moderate levels of additive noise. We envisage that our classifier will become a valuable resource for the zebrafish community.

Results & Discussion

Network Architecture

The architecture of KimmelNet is based on that described by Ishaq et al. (2017), which is in turn a simplified version of AlexNet (Krizhevsky et al., 2012). To reduce the number of trainable parameters (and change the nature of the network from classification to regression), we simplified the architecture further still (Fig. 1). The architecture consists of repeating units of 3 layers, consisting of 3 x 3 2D convolution, a Rectified Linear Unit (ReLU) and 2 x 2 max pooling. The output consists of a single fully connected layer, a dropout layer and a final fully connected (dense) layer consisting of a single output neuron. The total number of repeating convolution units, the number of convolutional filters in each and the dropout rate in the second-to-last layer were all determined using hyperparameter tuning, implemented using KerasTuner (Chollet et al., 2015).

Training KimmelNet

KimmelNet was trained on approximately 23,000 individual images of zebrafish embryos grown at 28.5°C, ranging in age from 4.5 to 51.75 hours post-fertilisation (hpf; Fig. 2A). We trained KimmelNet for 1,200 epochs as, for a greater number of iterations, the validation loss began to increase (while the training loss continued to decrease slightly), indicative of over-fitting. After 1,200 epochs, training loss was approximately 2.5 (Fig. 2B). Training took approximately 30 hours on the High Performance Computing (HPC) Cluster at the Francis Crick Institute (see Methods for hardware specifications).

KimmelNet's predictions are in close agreement with the Kimmel equation

We evaluated KimmelNet on two test datasets, one of zebrafish embryos grown at 28.5°C (23,180 images) and one at 25.0°C (16,530 images). Running KimmelNet on the 28.5°C dataset took approximately 160 seconds on the Crick's HPC hardware (see Methods), equating to just under 7 milliseconds per image.

Fitting an equation of the form $y = mx$ to the 28.5°C predictions resulted in a line with a slope equal to 0.999 (R^2 = 0.967) - a slope of 1.0 would indicate a perfect 1-to-1 correspondence between true hpf labels and predicted values (Fig. 3A). Saliency maps to illustrate pixel attribution, generated using an implementation of DeconvNet (Zeiler and Fergus, 2013), indicated that network predictions were driven by morphological features of the embryos, rather than any irrelevant features of the image background (Fig. 4).

The Kimmel Equation (Kimmel et al., 1995) states that

$$H_T = \frac{h}{(0.055T - 0.57)} \tag{1}$$

where H_T corresponds to hours of development at temperature T and h denotes the number of hours required to reach the equivalent developmental stage at 28.5°C. For the 25.0°C data, Equation 1 predicts that embryos will develop at approximately 80.5% of the rate of those grown at 28.5°C, equivalent to a straight line with slope 0.805. A straight line fit to the predictions produced by KimmelNet reveal a lower rate of development (slope = 0.760, $R^2 = 0.960$; Fig. 3A). However, the straight line corresponding to Equation 1 falls within the range of predictions produced by KimmelNet (Fig. 3A).

To determine how KimmelNet would perform on smaller test datasets, we took random samples of the data points shown in Figure 3A and fit lines to these points. Performing this sampling 10,000 times for either 20 or 100 random data points produced the range of line

fits shown in Figure 3B. From approximately 20 hpf, there is no overlap between the confidence intervals for the two test datasets. This demonstrates that KimmelNet can reliably distinguish between two populations of zebrafish embryos developing at different rates given just 20 images of each.

Plotting the residuals to the straight line fits showed that the mean prediction errors for both test datasets were distributed approximately uniformly about zero (Fig. 3C & D). For the 28.5°C data, the mean prediction error was 0.296 ± 0.016 h (mean $\pm$ s.e.m.) and the standard deviation was 2.394 h. For the 25.0°C data, the mean prediction error was 0.257 ± 0.016 h and the standard deviation was 2.018 h.

KimmelNet's prediction accuracy is not significantly impacted by moderate levels of additive noise

To test the general applicability of KimmelNet, we simulated further test datasets by adding noise at different levels to our raw test images (Supplementary Table 1). For low levels of additive Gaussian noise, the accuracy of KimmelNet's predictions was comparable to those on the raw test data (Supplementary Figure 1). However, for higher levels of Gaussian noise, combined with simulated non-uniform illumination, predictions produced by KimmelNet become unreliable (Supplementary Figure 2), with the image background playing a greater role in driving network predictions (Supplementary Figure 3). This indicates that while KimmelNet can be used successfully with noisier test data than that on which it was trained, there is an upper limit to how noisy the data can be.

Conclusion

The developing zebrafish embryo is used in many different types of studies and accurate staging is essential. When comparing an experimental group of embryos with a control

group, ensuring the embryos have reached the same developmental stage allows for meaningful comparisons to be made. KimmelNet enables rapid, unbiased assessment of thousands of images with minimal time commitment. We anticipate that our classifier will be a useful tool for the zebrafish community. There is also potential for KimmelNet to be useful in other developmental contexts, by retraining with images of other organisms.

Materials & Methods

Image Data

All images are the same as those used in a previously published study (Jones et al., 2022). Certain wells were manually excluded if it was apparent that the embryos died or were significantly out of view - the complete list of excluded wells is explicitly listed in the code (see github.com/djpbarry/KimmelNET/blob/main/train_model.py).

Data Partitioning and Augmentation

Image Data from Jones et al. (2022) corresponding to embryos grown at 28.5°C was split randomly 50:50 into training and test data on a well-by-well basis. Image data corresponding to embryos grown at 25.0°C was used exclusively for testing. Three data augmentation layers were prepended to the network for training purposes to prevent overfitting to the training data (Fig. 1). These consisted of random translation in the x-direction (max 5% of image width), random horizontal and vertical image flipping and a random contrast adjustment (maximum 10% adjustment of individual pixel values). Random translation in the y-direction was avoided due to the aspect ratio of the images (width > height) - any artifacts introduced by translation in the x-direction would be removed by the centre crop layer, but this would not be the case for translation in the y-direction.

Addition of Noise to Image Data

Noise was added to the original test image data to simulate different acquisition systems. Two different noise addition protocols were used (Supplementary Table 2). In the first, Gaussian noise with a standard deviation of approximately 1% of the maximum pixel value in the dataset was added to all test images. In the second, non-uniform illumination was simulated and Gaussian noise with standard deviation of 8% of the maximum measured pixel value was added.

Network Training

KimmelNet was trained on a single GPU node on the Francis Crick Institute's High Performance Computing (HPC) platform (Crick data Analysis and Management Platform - CAMP). Each node consists of 4 NVIDIA Tesla V100 SXM2 graphics cards. KimmelNet was trained on all 4 graphics cards simultaneously using the Tensorflow's distribute library. Training was performed using the Adam optimiser (Kingma and Ba, 2014) with a learning rate of 0.001 and mean squared error as the loss function.

Bootstrapping Test Predictions

To test the correspondence between predictions made by KimmelNet and those made by the Kimmel equation (Kimmel et al., 1995), test predictions were fit to a straight line through the origin of the form $y = mx$. To calculate confidence intervals associated with these fits, a series of 10,000 line fits were performed, each with a different random sample of the test data, consisting of either 20 or 100 data points. The maximum and minimum values of m produced by these line fits were then interpreted as a confidence range for the line fit to the complete data set.

Code Implementation

KimmelNet was implemented using Python 3.9 (www.python.org) and Tensorflow 2.9 (www.tensorflow.org). All plots in this manuscript were produced using Matplotlib 3.5 (Hunter, 2007) and all curve-fitting performed with SciPy 1.8 (Virtanen et al., 2020). Training and testing were performed with NVIDIA CUDA 11.2 (developer.nvidia.com/cuda-zone) and cuDNN 8.1 (developer.nvidia.com/cudnn).

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Data Availability

All training and test image data used in this study is the same as that described in Jones et al. (2022) and is available to download from the BioImage Archive (accession number S-BIAD531).

Software Availability

All code for building, training and testing KimmelNet is publicly available online (github.com/djpbarry/KimmelNET). A Binder implementation is also available to test KimmelNet on a subset of our test data (mybinder.org/v2/gh/djpbarry/KimmelNET/main?labpath=zebrafish_age_estimator.ipynb).

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Figure Legends

Fig 1. Illustration of the KimmelNet architecture. Augmentation layers active for training only are shown in blue.

Fig 2. KimmelNet converged to a training loss of approximately 2.5 after 1,200 epochs A. Example images of zebrafish embryos at various indicated stages of development - labels indicate hours post-fertilisation (hpf). Images were drawn from randomly selected wells in three different multi-well plates, constituting three different experiments. B. Plot of training and validation loss during training of KimmelNet with approximately 23,000 images similar to those shown in 'A'.

Fig 3. KimmelNet accurately quantifies developmental delay in line with Equation 1 A. Predictions produced by KimmelNet on two different populations of test data closely agree with those predicted by Equation 1. Each data point represents a single image. The solid lines represent the line of best fit (through the origin) to KimmelNet's predictions, while the dotted lines indicate the expected rate of development for the indicated temperatures based on Equation 1. B. Confidence intervals associated with lines of best fit illustrate that, even with just a small number of images, KimmelNet can discriminate between two different populations. The inner and outer confidence intervals indicate the range of lines of best fit determined for 10,000 randomly chosen sets of 20 (outer) or 100 (inner) data points from 'A'. C and D. Distribution of prediction errors produced by KimmelNet relative to lines of best fit (sold lines in 'A') and Equation 1 (dotted lines in 'A').

Fig 4. Saliency maps illustrate that KimmelNet responds to image pixels relevant to the zebrafish embryos Image background contributes little to predictions.

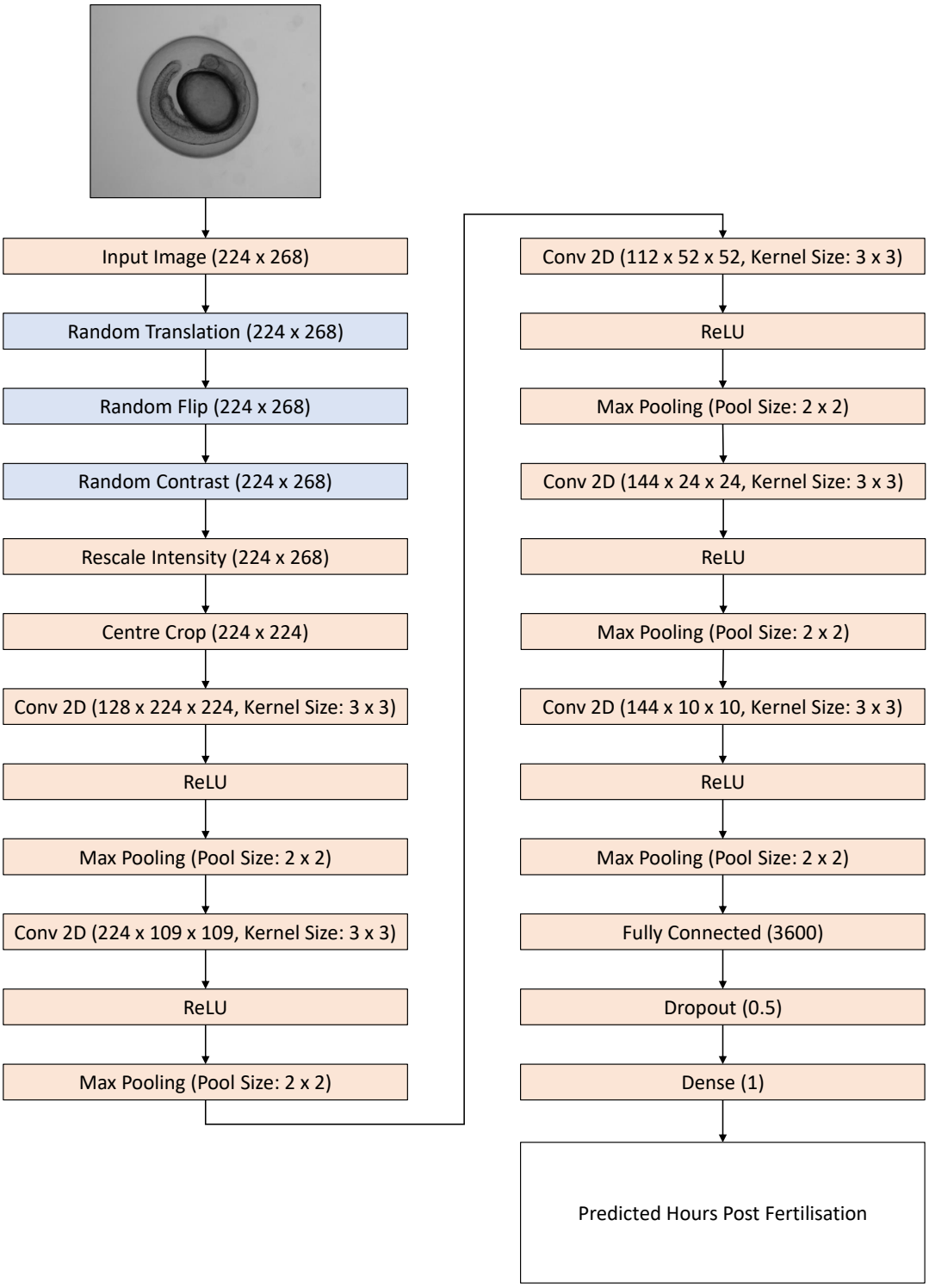
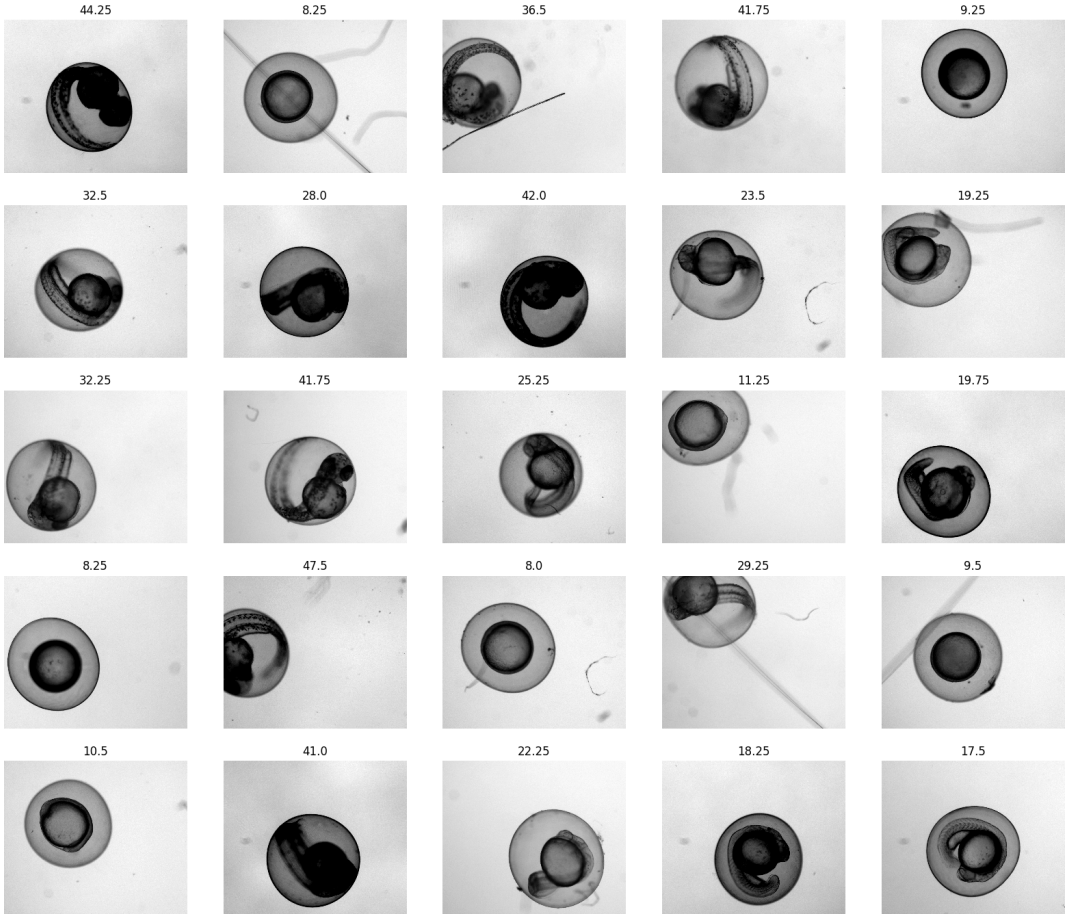

Input Image (224 x 268)
Random Translation (224 x 268)
Random Flip (224 x 268)
Random Contrast (224 x 268)
Rescale Intensity (224 x 268)
Centre Crop (224 x 224)
Conv 2D (128 x 224 x 224, Kernel Size: 3 x 3)
ReLU
Max Pooling (Pool Size: 2 x 2)
Conv 2D (224 x 109 x 109, Kernel Size: 3 x 3)
ReLU
Max Pooling (Pool Size: 2 x 2)
Conv 2D (112 x 52 x 52, Kernel Size: 3 x 3)
ReLU
Max Pooling (Pool Size: 2 x 2)
Conv 2D (144 x 24 x 24, Kernel Size: 3 x 3)
ReLU
Max Pooling (Pool Size: 2 x 2)
Conv 2D (144 x 10 x 10, Kernel Size: 3 x 3)
ReLU
Max Pooling (Pool Size: 2 x 2)
Fully Connected (3600)
Dropout (0.5)
Dense (1)
Predicted Hours Post Fertilisation

Figure 1

A.


44.25
8.25
36.5
41.75
9.25
32.5
28.0
42.0
23.5
19.25
32.25
41.75
25.25
11.25
19.75
8.25
47.5
8.0
29.25
9.5
10.5
41.0
22.25
18.25
17.5

B.

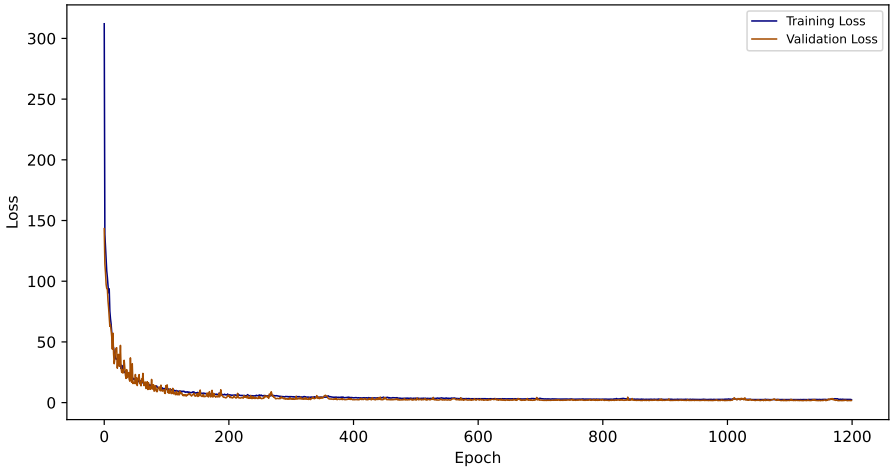

Training Loss
Validation Loss
Loss
Epoch
0
50
100
150
200
250
300
0
200
400
600
800
1000
1200

Figure 2

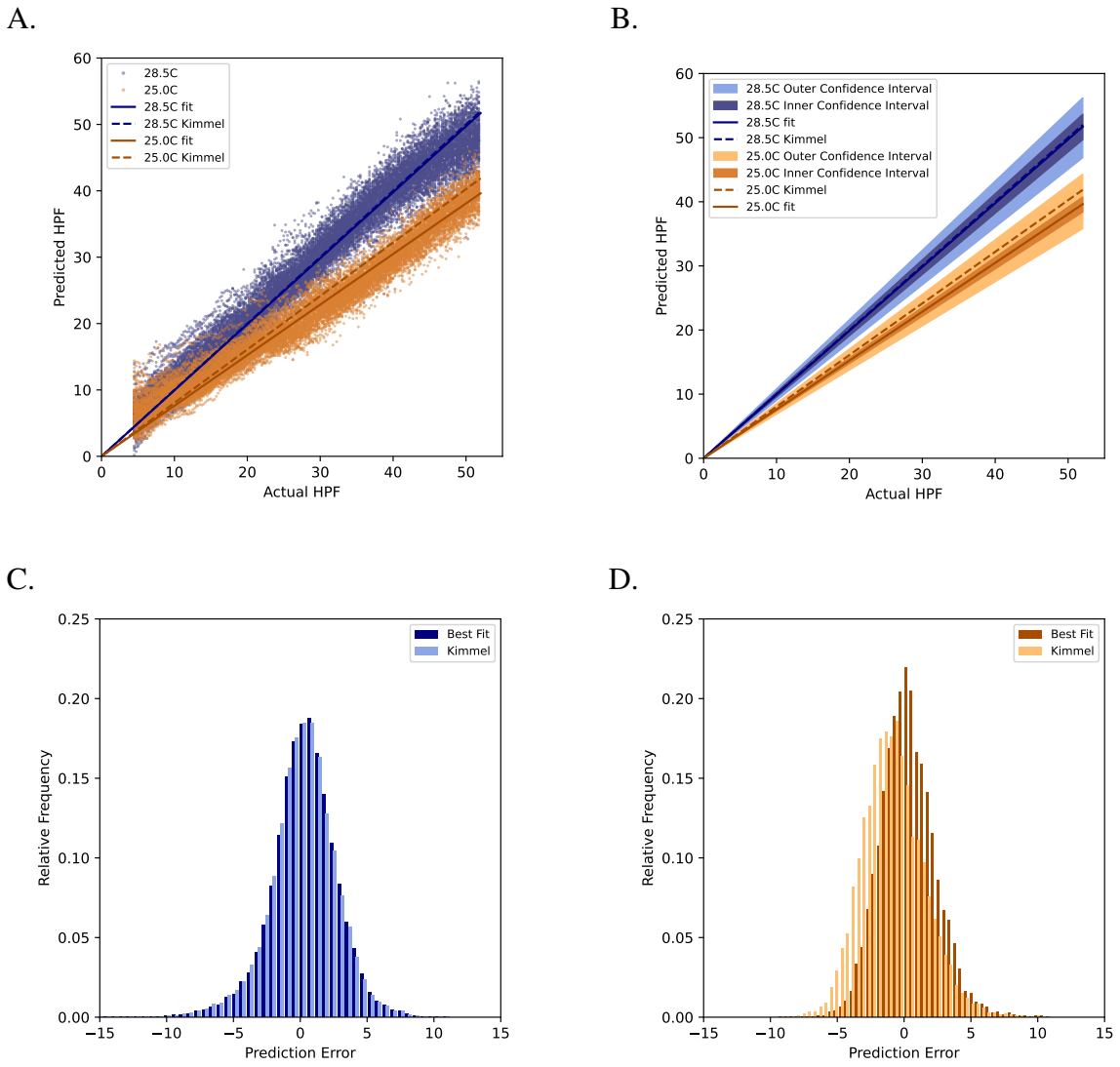

A.
28.5C
25.0C
28.5C fit
28.5C Kimmel
25.0C fit
25.0C Kimmel
Predicted HPF
Actual HPF
B.
28.5C Outer Confidence Interval
28.5C Inner Confidence Interval
28.5C fit
28.5C Kimmel
25.0C Outer Confidence Interval
25.0C Inner Confidence Interval
25.0C Kimmel
25.0C fit
Predicted HPF
Actual HPF
C.
Best Fit
Kimmel
Relative Frequency
Prediction Error
D.
Best Fit
Kimmel
Relative Frequency
Prediction Error

Figure 3

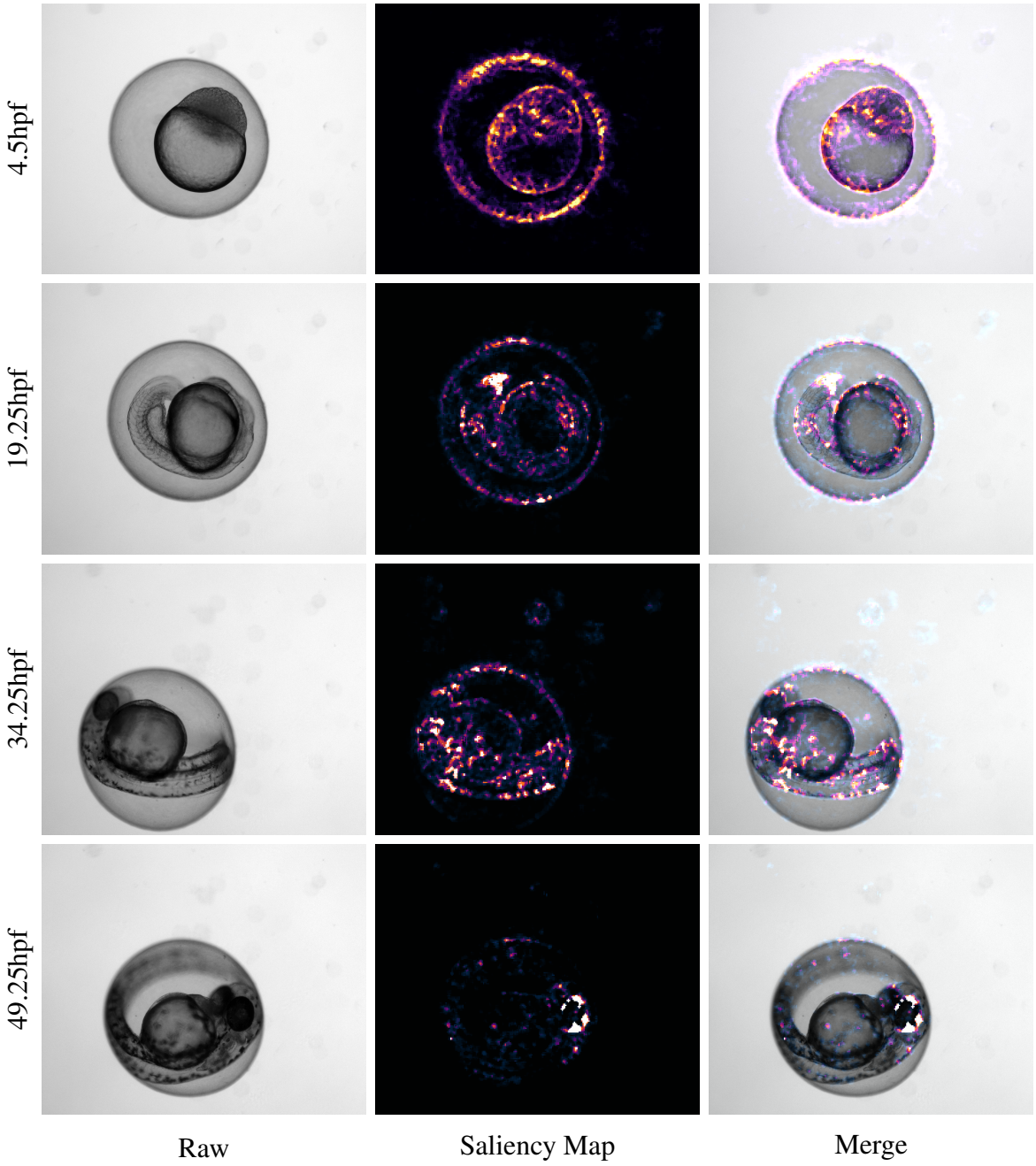


Figure 4