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Timekeeping by frog embryos, in normal development and after heat shock

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Summary

(1) Timekeeping refers to the uniformity of development in time. The precision of timekeeping is measured by the extent to which embryos, within an initially synchronous population, come to diverge in the course of their development.

(2) Divergence is measured as the variation in the stage of development reached between embryos allowed to develop for a fixed period of time. The lower the variation the better the timekeeping.

(3) Divergence among frog embryos that started development at the same time is hardly measurable after approx. 100 h of development. This striking uniformity indicates good timekeeping.

(4) Timekeeping is not impaired among the survivors following heat shocks that retard development and disturb and curtail morphogenesis.

(5) The immediate effect of heat shock is a stoppage of development, the duration of which is the same for all embryos in the same treatment batch. The embryos react to heat shock by rescheduling their development with the interpolation of a rest, the duration of which is controlled to the same precision as normal development. The postponement of development, without impairment of timekeeping, implies dis-engagement of the processes of morphogenesis from, and their subsequent re-engagement with, an enduring rate-determining activity unaffected by heat shock.

(6) We have searched for embryos whose rate of development was disturbed by heat shock to run slower or faster than the norm. We have found none. It seems that the (temperature-compensated) rate of development is invariant up to the moment of failure, or a change is immediately lethal.

Key words: amphibian embryos, timekeeping, heat shock, stress.

Introduction and methods

The integration of embryonic development clearly depends on the temporal coordination of individual processes. One result of this coordination is the repeatable correlation of the developmental stage of the embryo with time elapsed since fertilization. Anyone who works with naturally spawned amphibian eggs will be aware of the striking synchrony of their development. We decided, therefore, to use this material to investigate developmental timekeeping. In this paper, we characterize synchrony under natural conditions and demonstrate the stability of timekeeping under severe environmental stress.

Measurement of timekeeping requires the close staging of embryos. The choice of stage markers was governed by the following considerations. Stage markers should appear abruptly and their expression should be unequivocal. Criteria that depend on absolute size and precise shape are unsuitable as they may reflect differences in egg size, and vary with smooth deformations of the embryo. Trivial aspects of development may not be closely in step with major developments. Certain landmark events may occur earlier or later depending on conditions; for example, some fish hatch precociously at higher temperatures. The choice of stage markers is ideally confined to size-invariant, topological discontinuities that are essential to early development or later contribute essentially to the body plan.

We have used the appearance of the first cleavage furrow as a marker at the beginning of development and we have staged older embryos according to the number of somites segmented.

The first three pairs of somites segment at about the same time, thereafter segmentation is sequential. The interval between the segmentation of one somite and the next is a constant 140 min in *Rana* embryos

reared at 15°C. Accurate counts can be made on normal embryos with up to around 30 pairs of somites. Segmentation provides a series of some 27 well-defined and usable stages, of an equal duration, short in comparison with the duration of embryonic development. Somites were counted on fixed embryos stripped of their skin according to the procedure previously described (Elsdale, Pearson & Whitehead, 1976).

To study the robustness of timekeeping under stress, it is important to ensure that all the embryos in a batch receive exactly the same stress at exactly the same time. Ideally the stress employed should be a continuous variable, have instant ON/OFF and involve a treatment time short in comparison with the staging interval employed. Heat shocks of 10- to 40-min duration at 37°C approximate to these requirements.

Besides being a good stress for technical reasons, heat shock is an interesting agent in the context of timekeeping. Heat shock retards development and shocked embryos may come to lag nearly two days behind their controls.

Two considerations governed the choice of the developmental stage at which heat shock was given. Older embryos tolerate heat shock better than younger ones. There was also the need to minimize induced somite abnormalities that limit the accuracy and scope of somite counting. Shocks of 10-min duration usually induce a degradation of the segmental pattern sufficient to vitiate counting. There is a short period around the midgastrula, however, when heat shocks do not induce somite abnormalities (Elsdale & Pearson, 1979). Delivery during this refractory period enabled us to count somites in embryos that received 20-min shocks.

Somite counting was generally impossible in embryos that received shocks longer than 20 min. In order to measure timekeeping in these embryos external stage markers were employed. A series of ten stages, from the early neurula to the 7-somite stage, was defined from observation of normal embryos at 140-min intervals. The use of these stages is described in detail in the legend to Fig. 3.

Results

(A) *Timekeeping in normal development*

Three newly laid ovulations of *Rana temporaria* were collected from a pond close to the laboratory and samples of each were installed at 15°C within 30 min

Table 1. *Divergence in normal development and after heat shock*

(A) *Normal development*

	First count					Second count				
				Divergence					Divergence	
Ovulation	+h	n	$\bar{x}$	Somites	h at 15°C	+h	n	$\bar{x}$	Somites	h at 15°C
1	91	22	13·59	2·7	6·3	121	26	26·1	2·6	6·2
2	91	24	13·9	2·9	6·8	121	42	26·81	3·1	7·3
3	92	20	13·9	2·6	6·2	122	28	26·34	2·9	6·8
Repeat counts		135		2·6	6·2					

Somite counts were made on embryos from three ovulations (1, 2 and 3) developing normally at 15°C. The first counts were made 91 h after the first cleavage, when the embryos had segmented 14 somites. Another set of counts was made on embryos 30 h later, after the segmentation of a further 12 somites. Divergence is expressed in two ways: first, on the assumption that we are dealing with a normally distributed character, as four times the standard deviation of the somite count; second, as hours of development at 15°C, derived by multiplying the former divergence by 2·35, the time in hours between the segmentation of successive somites.

Repeat counts. To obtain a measure of counting error, counts were made on 27 embryos that had segmented from 19 to 27 somites. Each embryo was counted five times, being recoded before each count made so that the counter worked in ignorance. The variance of the counts for each embryo was computed and the pooled variance for all 27 embryos was calculated.

(B) *After heat shock*

	Number of embryos	Somites	h at 15°C
Control	53	2·6	6·2
HS20	47	3·3	7·7

HS20 embryos received a 20-min heat shock during the refractory period. Counts were made on heat-shocked embryos and untreated controls at the 17- to 18-somite stage and at the 30-somite stage. The entries in the table were calculated from pooled variances.

Table 2. *Retardation induced by heat shock*

Ovulation	Duration of heat shock (min)	No. of embryos shocked	Retardation: Somites-worth of time	Retardation: h at 15°C
1	10	10	4·5	10·5
	15	10	6·8	15·9
	20	20	8·7	20·3
	25	10	11·3	26·4
2	15	8	10·0	23·5
	25	8	16·0	37·3
3	20	30	8·0	18·7
4	40	102	16·0	37·3

The table includes data from four ovulations. Controls for each experiment were fixed and counted when they had segmented 27–30 pairs of somites. Heat-shocked embryos were fixed at the same time as their controls. Due to retardation, heat-shocked embryos had segmented fewer somites than controls. The difference between the mean somite counts for each pair gives the retardation in somites-worth of time. Multiplication by 2·35 (see legend to Table 1) converts to retardation in h of development at 15°C. Overall, 1 min of shock time resulted in roughly 1 h retardation. Note the differences in sensitivity between ovulations: a 25-min shock to ovulation 2 resulted in a markedly longer retardation than an identical shock to ovulation 1, and gave the same retardation as a 40-min shock to ovulation 4.

of collection. Cleavage began about 3 h later. All the embryos in an ovulation started to cleave over a 30-min period.

Table 1 presents divergences derived from somite counts made on samples from each ovulation at 91 h and 121 h after first cleavage. We define the divergence of a population as the range of stages that includes 95 % of the members and we use ± twice the standard deviation as a measure. We measured divergences of two or three somites, equivalent to 6 to 7 h. From repeat counts we obtained an estimate of the divergence attributable to counting error. The difference between the measured divergences of sibling populations and the divergence of repeat counts on the same embryos is too small for us to be certain that there is a real divergence in the course of development.

Essentially similar results were obtained using two ovulations collected from a different habitat.

(B) *Development after heat shock stress*

(1) *Heat shock induces retardation of development*

Table 2 documents an increasing retardation of development following shocks of increasing duration. Retardations were about 60 times longer than the duration of the inducing shocks. The table also demonstrates that the ovulations varied greatly in their sensitivity to heat shock.

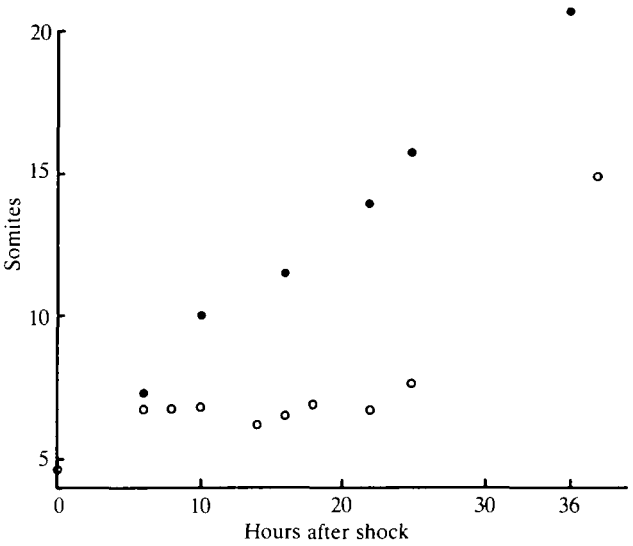


Fig. 1. Graph showing rest induced by heat shock. Embryos with 4–5 somites were shocked at 38°C for 17 min. Each control point (filled symbols) is the mean of two or three embryos; each experimental point (open symbols) is the mean of five to ten embryos. Segmentation ceased after about two further somites segmented at the normal rate. The graph illustrates the 16 h rest induced and the abrupt resumption of development that followed. The embryos were too damaged to allow a reliable measurement of the rate of resumed development, but see Table 3. Similar results from a 40-min shock to embryos 14 h younger are shown in Fig. 3.

(2) *Retardation is due to a rest followed by resumption of development at the control rate*

Fig. 1 plots the progress of development following a severe shock to 5-somite embryos. Two pairs of somites segmented normally after shock before development stopped, to be resumed some 16 h later. Not only somitogenesis was affected; development as a whole stopped. A similar result is seen in Fig. 3. The severe shocks used in these experiments meant that data on the rate of resumed development could only be obtained from a minority of the embryos.

Results after lesser shocks showed a resumption of development at the control rate in *Xenopus* and *Rana* (Pearson & Elsdale, 1979, fig. 10). Additional evidence is provided in Table 3 which shows that the rate of resumed development following a shock-induced arrest of 20 h is in good agreement with the control rate.

(3) *Timekeeping after 20 min heat shock*

Only exceptionally did embryos fail to hatch after a 20-min shock in the refractory period. These embryos, however, did not develop normally. They showed a variable degree of microcephaly and stunting, and sometimes a transient oedema. In fact a

Table 3. *Development is resumed at the control rate following heat shock*

		Time in h	Somite count n	Somite count $\bar{x}$	2nd count minus 1st count	Mean h/somite at 13°C
Control	1st count	0	23	17·17		
	2nd count	+44·5	20	30·6	13·43	3·3
HS20	1st count	0	17	18·24		
	2nd count	+41	39	30·97	12·73	3·2

Embryos received a 20-min heat shock during the refractory period. The induced retardation was worth nine somites or 21 h. Samples of control and shocked (HS20) embryos were prepared for counting at 18 somites, and further samples at 30 somites. Counts were made on the right sides of stripped embryos. The time in hours between the earlier count and the later, divided into the number of somites segmented during the same interval (second count minus first count) gives the mean rate of somite segmentation. At 13°C a somite forms about every 3·25 h. The measured difference in rate of somite formation between heat-shocked embryos and controls (approx. 5 min per somite) is within experimental error.

20-min heat shock is effectively lethal: out of 12 newly hatched larvae we reared only 2 to metamorphosis.

Table 1 shows that divergence was increased by about 1·5 h as a result of 20-min heat shock. This increase is not significant at the 0·05 % level ($F_{52}^{46} = 1{\cdot}54$). Sample sizes of the order of 250 would be required to determine whether an increase of this magnitude was significant. An increased divergence, furthermore, is expected after heat shock because the somites are more difficult to count. We conclude that, if timekeeping is affected at all after 20-min shock, the effect must be small and likely to require undue labour to confirm.

(4) *Timekeeping after 40-min heat shock*

A 40-min shock is about the most severe that can be usefully employed. The survival curve at the top of Fig. 3 shows that about a quarter of the embryos, in the experiment presented, disintegrated within a few hours of shock. Half of the shocked embryos were dead within 4 days. Fig. 2H–J illustrates the range of embryos that survived the immediate postshock period. The three embryos in Fig. 2H lost a large fraction of their cells by extrusion into the perivitelline space. These embryos died as arrested neurulae. Fig. 2I illustrates the four embryos that developed best: they were severely microcephalic, indeed one was almost acephalic. These were the only embryos, from this experiment, in which axial development was good enough to allow counts of the tail somites (see Fig. 2E). Fig. 2J shows embryos representative of the majority. These embryos show very poor axial development; segmentation was inhibited or partial and grossly abnormal. The pictures and the survival curve attest to the exceedingly injurious effects of 40-min shock, and also illustrate the highly variable morphogenetic response on the part of individual embryos from the same ovulation.

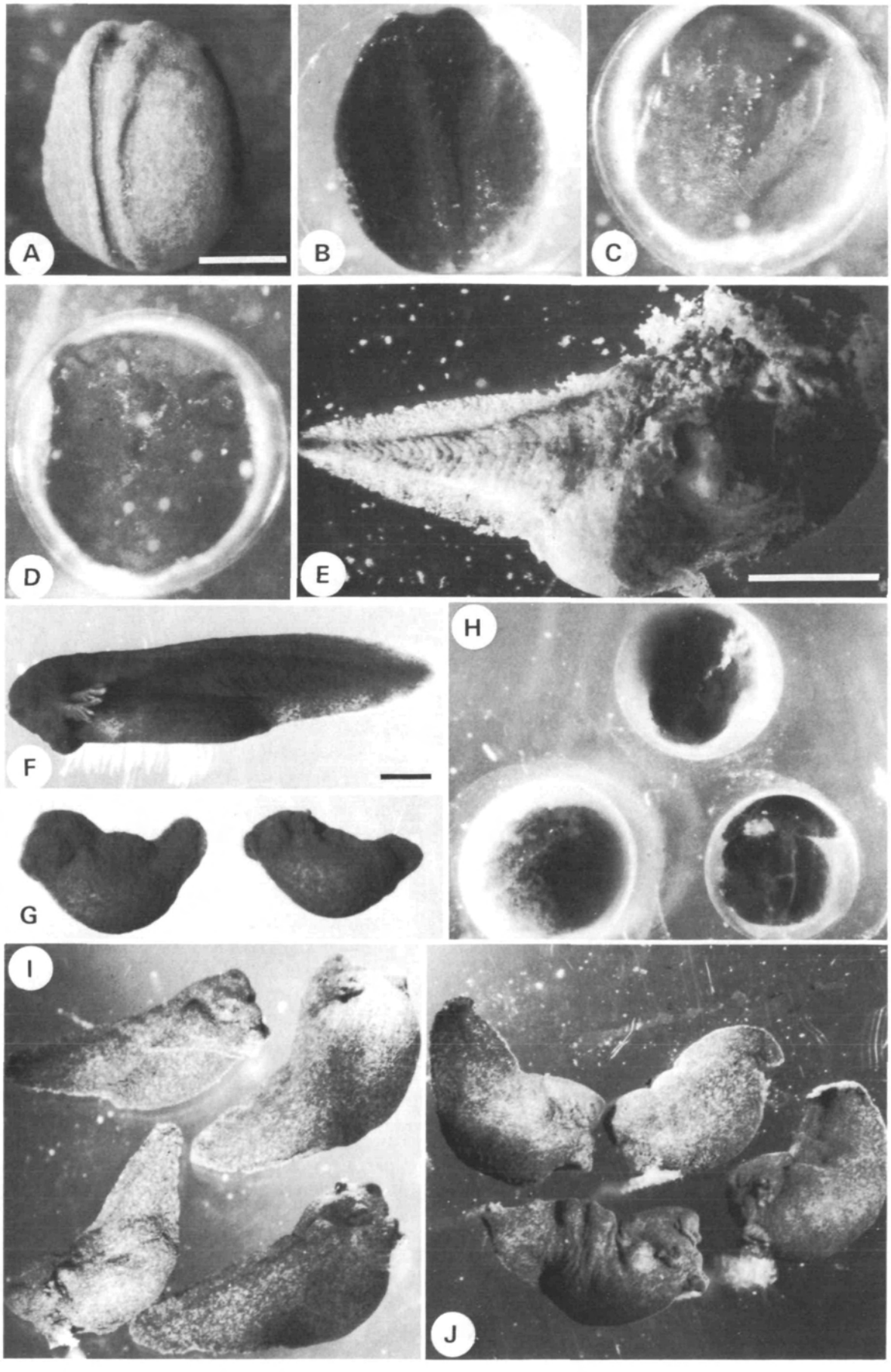
Turning to timekeeping, the lower part of Fig. 3 graphs the progress of shocked embryos and their controls. A rest of 37 h was induced. Although embryos were arrested as early neurulae, the mechanics of neurulation were concluded essentially normally (Fig. 2A–C). The figure shows that, using the external stage markers described in detail in the legend, heat-shocked embryos at no time ranged over more than two stages. The countable embryos fixed on the 3rd and 4th days suggest a resumption of development at the normal rate, but it should be borne in mind that they are unrepresentative. The

Fig. 2. Morphology of control and heat-shocked embryos. Bars, 1 mm. The bar on A refers also to B–D. The bar on F refers also to G–J.

(A–D) Neurula stages; (A) normal embryo, neural folds closing, jelly and vitelline membrane removed; (C,D) two living embryos, one stage, 2·35 h, younger than A, photographed within their jelly coats during the rest induced by heat shock. In C, early neurula, the neural field is viewed from above, the presumptive head is top right; the thick, oedematous folds are beginning to rise anteriorly. Some 15 cells have been extruded from the neural plate. Embryo D is viewed from below to show the two unusual knob-like extensions marking the anterolateral extremity of the head folds. Embryo B also received a heat shock and is some 20 h older than the control embryo A. Apart from the slightly lagging, oedematous head fold on the right side, the appearance is the same as embryo A.

(E,H–J) 3·5 days after 40-min shock. The three embryos in H are moribund following massive extrusion of cells into the perivitelline space. The four embryos in J are typical of the majority in the experiment. I shows the four embryos that tolerated heat shock best. These four embryos alone had countable tail somites and one with 21 tail somites is shown stripped in E. The first tail somite is taken to be the segment immediately posterior to the anal marker, not clearly seen on this photograph.

(G) Two examples from the repeat experiment in which embryos were more resistant to heat shock. These embryos and a control in F show no oedema and therefore appear smaller than embryos in I,J although the magnifications are the same.


A
B
C
D
E
F
G
H
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J

experiment shows that timekeeping remains virtually unimpaired under the most severe assault.

A replicate experiment was performed on embryos from a different ovulation which gave a lesser retardation of 26 h for the same shock. The result was otherwise essentially the same as before. Fig. 2G shows two representative, heat-shocked embryos from this experiment. The embryos have no oedema and hence appear smaller and darker than the highly oedematous embryos from the first experiment.

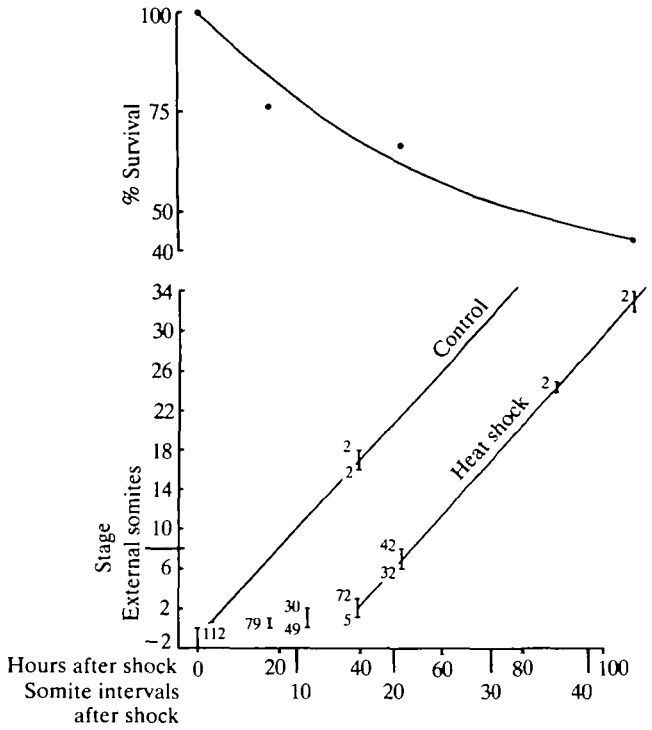

Fig. 3. Survival and progress of embryos after 40-min heat shock. 112 embryos were given a 40-min heat shock at the late gastrula stage. The upper figure plots survival. About half of the embryos survived no longer than 4 days. The lower figure plots the development of the survivors of heat shock and controls. Stage of development is marked along the vertical axis. The numbers above the bar at 8 on the vertical axis represent numbers of somites. External stages are represented below the bar, and have been arbitrarily numbered in sequence with the numbers above. At the time of shock embryos were at stage −1 and stage 0. The former is a transitional stage between the late gastrula and the neural plate. Stage 0 is the neural plate, characterized by a slit blastopore and sinking yolk plug, and a slightly raised neural plate. 18 h after shock, 79 embryos survived and all were judged to be at stage 1, the early neural fold stage (Fig. 2C). At 27 h, 49 embryos remained at stage 1 and 30 embryos had developed to stage 2, the midneural fold stage at which the folds have started to rise and approach the midline. At 40 h, five embryos remained at this later stage and 72 had reached stage 3, neural closure, with folds touching in the trunk region, but not yet over the head. The embryos in Fig. 2A,B are between stages 2 and 3. At 50 h, 42 embryos showed fusion of the folds over a part of their length. Fusion normally begins on the 7-somite embryo. 32 embryos were not younger than stage 5 (closure complete) and not older than stage 6 as fusion had not commenced. Two of the four embryos in Fig. 2I were fixed at 86 h and both had 24 somites.

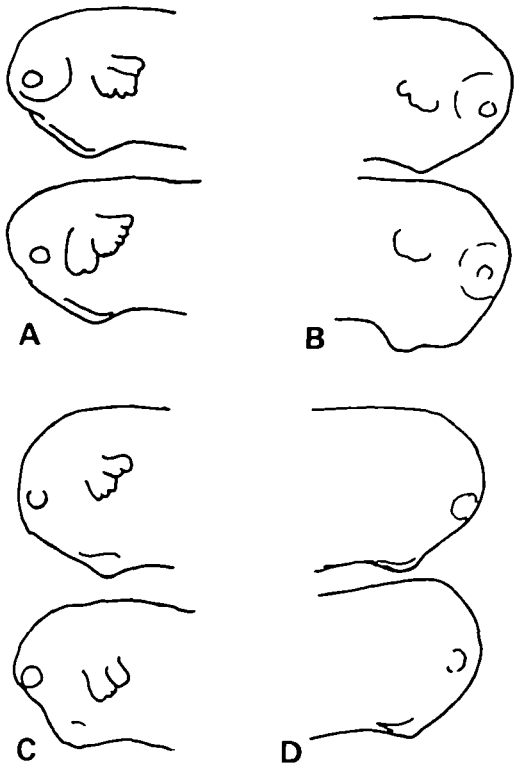

Fig. 4. Induced thermotolerance results in a reduction in the duration of rest induced by heat shock. *Camera lucida* drawings from photographs of fixed embryos: A is to be compared to B, C to D. (A) Embryo received a 2-min shock at the neural fold stage. One day later, A and B received an 8-min shock around the 10-somite stage. The embryos were fixed 2 days later. Gill development indicates the relative developmental stage of the embryos. Although A actually accumulated more time at 37 °C than B it is clear that the gills in A are more advanced in their development than those in B. The 2-min shock given to A has had a protective effect and reduced the effectiveness of the subsequent 8-min shock. B was not so protected and the 8-min shock has had a greater effect. 8-min shocks do not inhibit gill development. Induced thermotolerance is here seen as a reduction of the duration of the rest induced by the second shock. A similar result is given by comparison of C and D. (C) The embryo received an 8-min shock and, 2 days later, received a second 8-min shock, while D received a 16-min shock. The total length of time at 37 °C was the same for both batches. Embryos were fixed 2 days later. The 16-min shock caused the gills to develop abnormally. The drawings show that at the time when the gills in C were well into their development, the gills in D had not yet started to develop.

(5) *Thermotolerance*

We demonstrate that the duration of the rest induced by heat shock is reduced following a prior shock (see Fig. 4). Reliance cannot be placed on somite counting because of uncertainties in counting zones of abnormal segmentation. We have therefore used the development of the gills as an indicator of developmental stage for this case.

Discussion

(A) *Interpretation*

Stable timekeeping

The measured divergence among normal siblings after 90–120 h of development is 6–7 h. Counting error arises where there is a doubt concerning which segment is to be judged the last well-formed somite. Measured counting error is about the same as the measured divergence. Our results suggest that if there is a genuine increase in divergence, during the first approx. 100 h of development, it is small and likely to be appreciably less than the measured 6–7 h. It would require counts on hundreds of embryos to confirm such a marginal increase.

Following 20-min heat shock measured divergence is increased by about 24 % (95 % confidence limits: 6 % and 64 %). This measured increase is short in comparison with the retardation induced by shock. We have earlier given reasons why we think it is an overestimate. We conclude that if there is an increased divergence after a 20-min shock, it is negligible in the context of timekeeping.

The credibility of the result after 40-min shock depends on the validity of the employment of external stage markers. We have earlier expressed our preference for discontinuous markers. In this experiment we had to work with continuous change. The scores, therefore, could have been biased by smooth deformations depending on, for example, the degree of relaxation of the vitelline membrane, oedematous swelling, etc. Within a single treatment batch of sibling embryos such factors, however, could be expected to exaggerate variation rather than bias toward uniformity. Heat-shocked embryos became arrested at the wide neural fold stage and indeed presented a significant deformation of the normal appearance (Fig. 2C,D). By the next stage, however, with rising folds approaching the midline, the appearance was almost normal (Fig. 2A,B). Our observations corroborated previous experience that neurulation is not inhibited in the survivors of heat shock, except where there is considerable loss of cells (Fig. 2H). We conclude that our results can be taken at their face value to demonstrate that timekeeping remains unimpaired after heat shock, up to the limit of destruction testing.

Invariant timekeeping

The maintenance of synchrony among severely heat-shocked siblings demonstrates the stability of timekeeping. Only when contrasted against an otherwise variable response to heat shock, however, is the invariance of timekeeping shown to be exceptional.

The injurious effects of heat shock are expressed through survival, retardation and morphogenesis. We itemize the three sources of variation in these indicators.

(1) Between sibling embryos of the same treatment batch. Length of survival and malformation vary (Figs 2, 3). Retardation, here but not in 2 and 3 below, is a measure of timekeeping and does not vary (Table 1).

(2) Between batches of siblings given shocks of different durations. There is variation in all three indicators. For retardations see Table 2.

(3) Between samples from different ovulations given the same treatment. All indicators vary; for retardations see Table 2, for morphogenesis see Fig. 2.

(B) *Implications*

Lindsley & Poodry (1977) have reported heat-shock-induced arrest of development in *Drosophila*. Others have noted, with less precision, that development is slowed down or delayed following heat shock.

Detailed examination has disclosed that the retardation of development induced by heat shock is the result of a controlled developmental rest followed by a resumption of development at the normal rate. The clarity of this result and the failure to alter the rate of resumed development, makes us wonder whether an underlying pattern of rest and resumption may be universal in cases of developmental retardation, however caused. 'Underlying' because allowance has to be made for development already 'in the pipeline': in the frog some three pairs of normal somites are segmented at the normal rate following shock, before development halts.

Certainly, our results point away from the idea that the temperature-compensated rate of development is a continuous variable. In fact, they point in the opposite direction and suggest that development knows only two settings, stop and go, where the latter implies a single, fixed and predetermined rate.

Orderly response to heat shock

The duration of the rest that we induced by heat shock is precisely the same for all viable embryos within the same treatment batch. If the cessation of

development were due to the widespread and indiscriminate effects of heat shock within the embryo, it would be difficult to understand this result, for the degree and pattern of such damage would be expected to vary somewhat from embryo to embryo, as would their ability to cope. In this scenario, incorporating a chance element, embryos would be expected to resume development at different times, and postshock synchrony of siblings would be lost.

Heat shock cannot be just a spanner in the works. It elicits an orderly response in which the rest is an interpolated stage subject to a temporal control as strict as that pertaining throughout normal development. The duration of the rest shows the same kinetic response to rearing temperature as normal development (Cooke & Elsdale, 1980). This behaviour is inconceivable were the rest merely a period of profound inactivity. We infer from the maintenance of good timekeeping that the pattern of rest and resumption is the adaptive response by a mechanism that has remained undamaged. We are led to believe that an active characteristic of normal development continues throughout the rest and thereafter.

There are two aspects of the behaviour of embryos after heat shock, the postponement of normal development and the timekeeping aspect.

Postponement of normal development. With longer shocks we witness progressively deficient morphogenesis. The postponement of development after shock takes little account of the embryos' diminished potential for morphogenesis. One has a picture of a lamed morphogenesis led along at the normal rate regardless. There is further evidence that postponement and morphogenesis are independent: their responses to rearing temperature are different. The survival and morphogenesis of heat-shocked embryos can be dramatically improved by rearing postshock at low temperatures, approx. 6°C (Cooke & Elsdale 1980). In contrast, the duration of the rest is temperature compensated in the same manner as normal development. These results are explained if we assume that rescheduling is immediate and irrevocable, whereas morphogenesis reflects the extent of repair taking place during the rest.

Thermotolerance is increased following heat shock and the induction of heat-shock proteins (HSPs) (Gerner & Schneider, 1975; Schlesinger, Ashburner & Tissieres, 1982). Induced thermotolerance has been measured in the frog. The length of the zone of abnormal segmentation induced by a second heat shock is only half of the length of that induced by an identical single shock delivered to siblings at the same stage (Elsdale & Pearson, 1979). Here we show that the duration of the arrest following heat shock is reduced following a prior shock. The shocks that we have applied can certainly be expected to induce the synthesis of HSPs in *Rana* (Bienz, 1982). Is it possible that the duration of the rest and the maintenance of temporal control over development in *Rana* depend on the selfregulated synthesis of a subset of HSPs? This hypothesis may be amenable to experimental test by manipulation of the HSP genes or their products.

Embryos from different ovulations, and hence of different parentage, differ in the length of the rest induced by a standard shock. This may provide a natural situation in which to look for differences in components of the control mechanism. Our results suggest that the duration of the rest is an inherited character. It would be useful to know if this character showed maternal inheritance.

Timekeeping: mechanisms. The mechanistic basis of timekeeping is not clear. We cannot say, on the basis of our results, whether the embryo is a formal unity and good timekeeping the immediate dividend of structural and physiological complexity, or whether the embryo is a radical duality, a locatable timekeeper standing outside of development.

One could imagine, for instance, a situation whereby heat shock caused the destruction of a substance X with consequent cessation of development until X had been replenished up to some threshold. This example belongs to the class of mechanisms called relaxation oscillations. Were such an oscillation to exist, our experiments indicate that it should possess an exceptional stability. Indeed, this hypothetical oscillation would be, perhaps, the most stable entity in the embryo. The oscillation would behave, furthermore, reliably in the face of temperature shock, until the moment it suffered a catastrophic failure.

Our results suggest that morphogenesis depends on the constancy of some underlying process. This process is the *sine qua non* for development and the life of the embryo. In the same way that morphogenesis may be abnormal due to interference that leaves the genome unaffected, so, we infer, heat shock may disturb morphogenesis but leave the rate-determining process unaffected. This inference suggests a restriction analogous to the central dogma of molecular biology. The parallel between the genome and the basis for timekeeping leads us to wonder whether the latter is part of the former, and whether the exceptional stability of DNA may underpin an embryonic 'timekeeper'.

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