Why Is *cardiac* Lethal?

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The cardiovascular system is one of the first systems to begin functioning during vertebrate development. In the axolotl, the heart begins to beat at stage 35 (Bordzilovskaya *et al.*, 1989), and circulation is established 1-2 days later.

The early onset of cardiac function is widely assumed to be necessary for the continued survival and development of the embryo. However, it is also known that amphibian embryos and larvae can survive for extended periods without a heart (Knower, 1907; Copenhaver, 1926; Copenhaver, 1955) or, in the case of *cardiac-lethal* (*c/c*) mutants,with a non-functional heart (Humphrey, 1972). So, the question remains: why does the heart begin to beat so early?

One obvious possibility for the early onset of heart function is for gas exchange. However, in a recent study, we demonstrated that oxygen consumption was the same in wild-type embryos, embryos from which the pre-cardiac mesoderm had been surgically removed (*i.e.*, *cardiac* phenocopies), and embryos treated with carbon monoxide (Mellish *et al.*, 1994), even under severely hypoxic conditions. Oxygen consumption was also the same for *c/c* embryos, demonstrating that the *c* gene does not affect gas transport (Mellish, Smith and Pinder, manuscript submitted). These results raise the additional question of why the *cardiac-lethal* mutation is lethal. If early cardiovascular function is not required for gas exchange, what eventually kills *c/c* larvae?

Among the possibilities suggested in our previous studies are that early blood circulation is necessary to maintain fluid and/or ionic balance, and/or excrete nitrogenous wastes through early renal function. This is particularly plausible given the edema observed in *c/c* embryos (Humphrey, 1972). A second possibility is suggested by Smith and Armstrong's (1993a, b) demonstration that the inability of *c/c* embryos to feed is not linked to the absence of circulation. Thus, *c/c* embryos may simply starve to death.

Materials and Methods

To examine these possibilities, the pronephric anlagen were bilaterally removed from a series of stage 26 wild-type embryos using standard microsurgical techniques (Asashima *et al.*, 1989). Post-surgically, the flaps of ectoderm which covered the pronephra were replaced and allowed to heal. Embryos were raised in separate agar-lined 24-well tissue culture dishes in 25% Holtfreter's solution at 18°C. Embryos were examined daily, and the medium was changed every second day. After hatching, larvae were kept in separate wells of six-well tissue culture dishes.

In a second experiment, wild-type embryos were allowed to remain unfed. These embryos were the siblings of both the pronephros-less (PN^-) embryos, described above, and *c/c* embryos. As above, all embryos were raised separately in 25% Holtfreter's medium at 18°C in 24- and then 6-well dishes. By maintaining larvae separately, cannibalism was avoided. In this way, the effects of the absence of kidney function, and starvation, could be compared to the congenital absence of blood circulation caused by the *c* gene.

Results and Discussion

Starvation as a cause of death. The time of death from each cause was determined (Figure 1). *Cardiac-lethal* embryos died significantly earlier than unfed wild-type larvae. At first, this result suggests that starvation cannot be the cause of death in *cardiac-lethal* embryos. However, there is a caveat to this interpretation.

By the open mouth stage (44) wild-type larvae had resorbed all yolky endoderm. As starvation progressed, they began to resorb their gills and, to a lesser extent, their tail fins. In contrast, *c/c* embryos were apparently unable to utilise all of their stores of yolky endoderm, which could be easily observed through the abdominal wall even in older *c/c* larvae (see also (Humphrey, 1972). As well, although their gills remained characteristically underdeveloped (Humphrey, 1972; Smith and Armstrong, 1993b), there was no obvious resorption of either these organs or the tail fins as *c/c* embryos aged.

Therefore, although *c/c* larvae died earlier than unfed wild-type larvae, starvation cannot be completely discounted as a contributing factor in their death. In addition to being unable to feed (Smith and Armstrong, 1993a, b), the absence of circulation appears to prevent *c/c* larvae from fully utilising available nutrients. Thus, even if *c/c* larvae were able to feed, they might starve due to their inability to distribute nutrients.

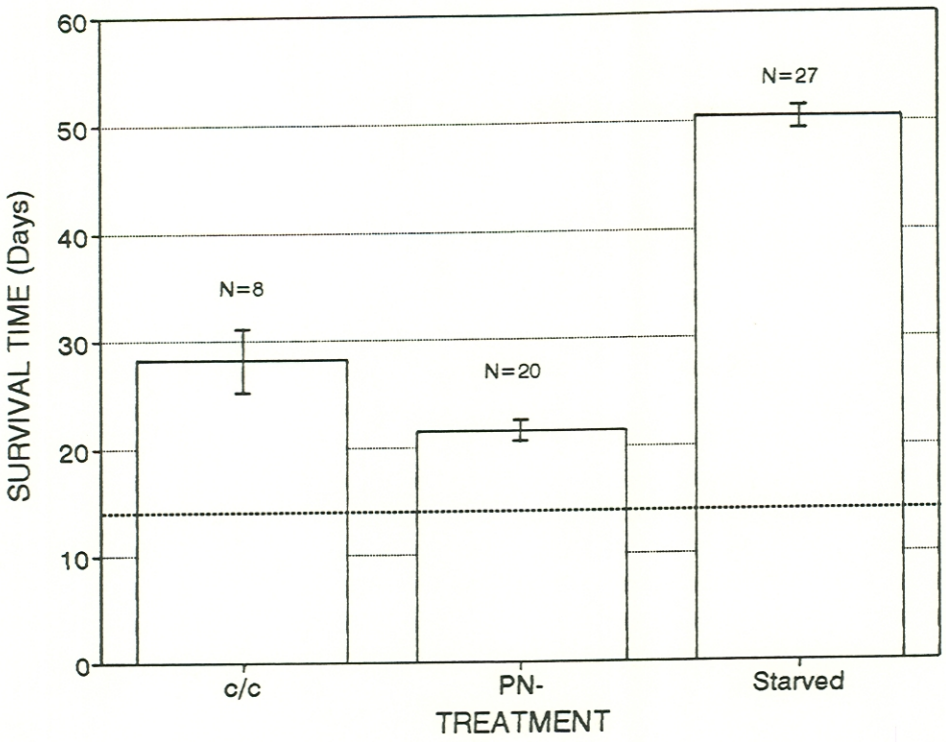

Figure 1: Survival time (in days ± SE) of *cardiac-lethal (c/c)* pronephros-less (PN^-), and unfed wild-type (starved) axolotl larvae. All times are after stage 35, at 18°C. For reference, the approximate time of hatching is indicated by the solid line at 11 days.

Lack of pronephric function as a cause of death. Another suspected contributing factor in the death of *c/c* larvae is the lack of renal function. To test this possibility, the pronephric primordia were bilaterally removed from 20 stage 26 embryos. Such PN^- embryos developed without pronephra. In all cases, the embryos developed functional hearts and blood circulation at the expected time.

Pronephros-less larvae died an average of 1 week earlier than *c/c* controls (Figure 1). Therefore, lack of pronephric function cannot be the cause of death in *c/c* larvae, since PN^- larvae (which have no pronephric function) die sooner.

In addition, observations of the gross morphology of PN^- larvae revealed that PN^- larvae became markedly more swollen than their *c/c* counterparts (Figure 2). The swelling was so excessive that, in the later stages, blood circulation was partially or completely arrested, presumably due to the pressure exerted on the cardiovascular system by the fluid accumulated in the thoracic cavity.

Cardiac-lethal larvae do become edemic, but not to the same extent. This leads to the surprising conclusion that some pronephric function must be present in *c/c* larvae *despite the absence of blood circulation*. It is unclear how this could occur, but given that *c/c* larvae become edemic (albeit less so than their PN^- counterparts), it suggests that swelling occurs until a critical pressure is reached, after which some of the accumulating fluid can be expelled through the pronephra. We are currently investigating this possibility.

If this is the case, and edemic fluid is expelled simply due to increased pressure, *c/c* larvae could still suffer from fluid and/or ionic imbalances, possibilities which we are also investigating.

Conclusions

What eventually kills *c/c* larvae remains unclear. From the results presented above, starvation (due to the inability of *c/c* animals to feed) is unlikely to be the cause of death, since mutant animals die much sooner than starved wild-type siblings. However, the restricted ability to mobilise nutrients may be a contributing factor. Similarly, the complete absence of renal function is unlikely to be the sole cause of death, although an inability to maintain ionic balance (further exacerbated by the absence of cutaneous circulation) could well be a contributing factor.

We have previously reported that the absence of circulation does not impair gas transport (Mellish *et al.*, 1994; Mellish, Smith and Pinder, submitted). However, these studies were expressly done on stage 39 embryos, before the edemic swelling became too severe.

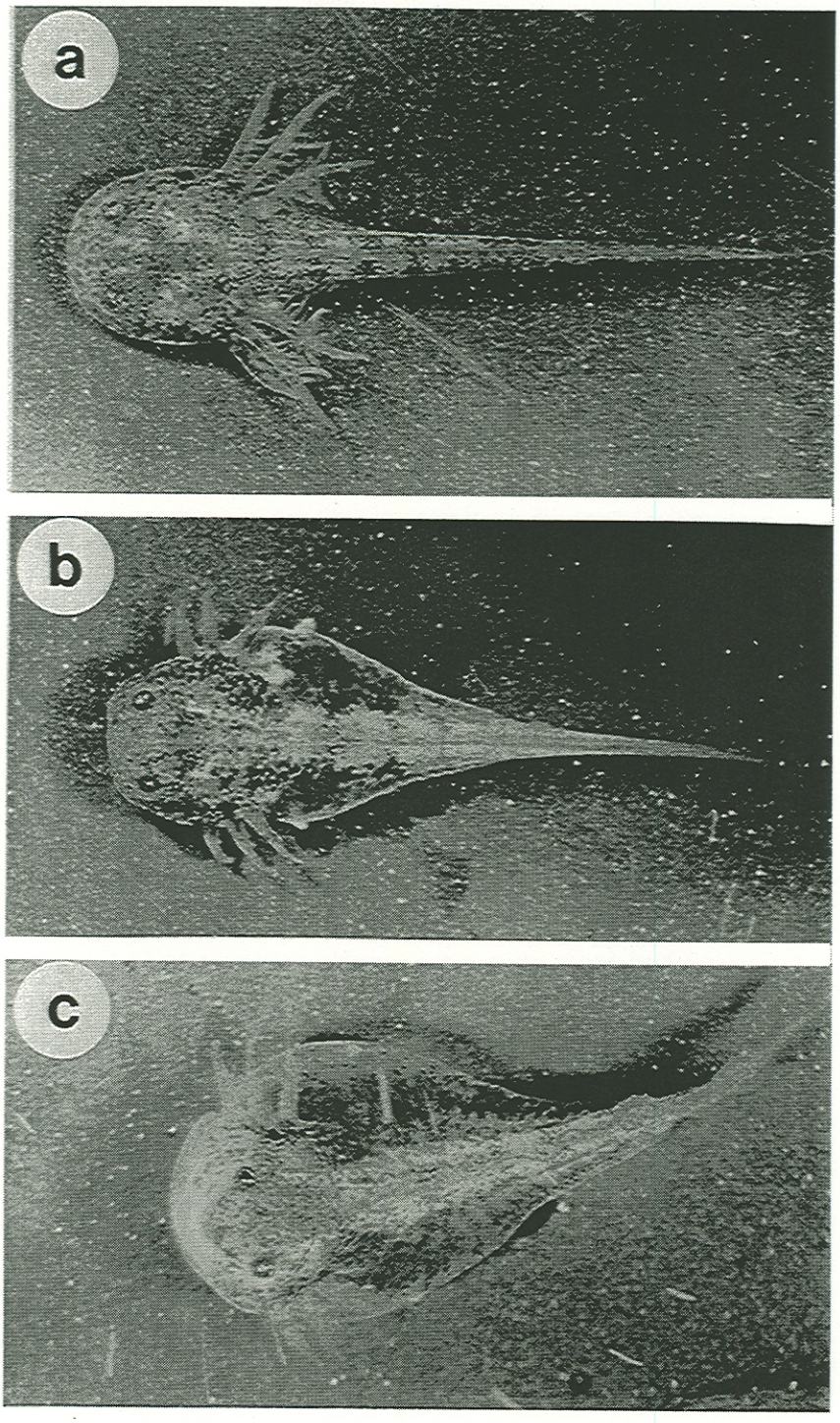

Figure 2: Photomicrographs of wild-type (a), *c*/*c* (b), and PN$^-$ (c) hatchling larvae. Although *c* mutant larvae acquired their characteristic pear shape, PN$^-$ larvae became much more severely swollen. Eventually, circulation arrested as the larvae became almost spherical in shape. Such larvae usually burst prior to death.

It is distinctly possible that the absence of circulation may impair gas transport once *c/c* larvae become more severely edemic, given the increased diffusion distance.

Thus, the physiological role (if any) of early cardiovascular function remains unknown. By examining what eventually kills *c/c* larvae, we may to be able to determine the time during development when various functions of the circulatory system become physiologically essential.

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