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Cover:

Color varieties of the Chinese Giant Salamander (*Andrias davidianus*) from aquaculture farming operations in China. *Photo Sumio Okada*.

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Survey techniques for giant salamanders and other aquatic Caudata

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Abstract.—The order Caudata (salamanders and newts) comprise ~13% of the ~6,800 described amphibian species. Amphibians are the most threatened (~30% of species) of all vertebrates, and the Caudata are the most threatened (~45% of species) amphibian order. The fully aquatic Caudata family, the Cryptobranchidae (suborder Cryptobranchoidea), includes the the world's largest amphibians, the threatened giant salamanders. Cryptobranchids present particular survey challenges because of their large demographic variation in body size (from three cm larvae to 1.5 m adults) and the wide variation in their habitats and microhabitats. Consequently, a number of survey techniques (in combination) may be required to reveal their population and demography, habitat requirements, reproduction, environmental threats, and genetic subpopulations. Survey techniques are constrained by logistical considerations including habitat accessibility, seasonal influences, available funds, personnel, and equipment. Particularly with threatened species, survey techniques must minimize environmental disturbance and possible negative effects on the health of targeted populations and individuals. We review and compare the types and application of survey techniques for Cryptobranchids and other aquatic Caudata from a conservation and animal welfare perspective.

Key words. Survey techniques, giant salamander, amphibian, Caudata, Cryptobranchid, conservation

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Introduction

Amphibians are suffering from one of the greatest rates of decline and extinction of any vertebrate class. One of the most unique, iconic, and threatened amphibian clades in the Caudata are the fully aquatic Cryptobranchids (family Cryptobranchidae; suborder Cryptobranchoidea). All three Cryptobranchids are fully aquatic and include the world's largest amphibians: the Critically Endangered, Chinese giant salamander (*Andrias davidianus*), the Near Threatened, Japanese giant salamander (*A. japonicus*), and the North American giant salamander (*Cryptobranchus alleganiensis*), commonly known as the Hellbender (CNAH 2011).

The conservation potential of Cryptobranchids extends beyond their immediate conservation needs. As iconic species, Cryptobranchids offer an ideal opportunity to develop public awareness and government and institutional support for water catchment management. In Japan, *A. japonicus* has become a national symbol, attracting publicity including parades with large floats, education and environmental awareness campaigns, and village conservation programs. Similarly, in the People's Republic of China, the release of *A. davidianus* from farm stock has received widespread government support and formal public recognition, and this species is becoming a symbol for watershed conservation. There is also an increasing momentum toward establishing *C. alleganiensis* as an icon for watershed conservation in the USA (Browne et al. 2012a, b).

However, in addition to public and government support, the conservation of Cryptobranchids and other aquatic Caudata relies upon scientific knowledge of their conservation genetics, population demography and size, habitat and microhabitat variables, reproduc-

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Figure 1. *Andrias davidianus* is the largest and most threatened Cryptobranchid, and can reach 200 cm in total length and 59 kg in weight. *Image Robert Browne.*

tion and life stage survival, and environmental threats. The most appropriate survey techniques to achieve this knowledge will depend on survey objectives in concert with logistical constraints including the type of habitat surveyed (Dodd 2009). The choice of survey techniques must consider interacting factors, including the species' autecology, targeted life stages, and season, as well as water depth, velocity, and clarity (Dodd 2009). Survey techniques must minimize environmental disturbance and possible negative effects on the health of the targeted individuals and populations through the spread of pathogens and trauma to individuals.

The conservation needs of Cryptobranchids vary widely between the three species. *Andrias davidianus* was until recently considered almost extinct in nature. However, recent evidence shows that there are a number of relict populations distributed throughout China. The few remaining populations (in lowland areas) are fairly genetically homogenous, probably due to anthropogenic transport and the building of canals over China's ~6,000 year history of civilization. Nevertheless, there are genetically distinct populations remaining (Tao et al. 2005), and ongoing molecular studies may reveal even finer population structure (R. Murphy, pers. comm.) and further Evolutionarily Significant Units (Crandall et al. 2000).

Andrias davidianus has a considerable aquaculture potential, and more than 1000 licensed aquaculture facilities are in production in China with up to 106 individuals in stock. In concert with aquaculture, there are an increasing number of restocking programs using aquaculture brood stock. However, aquaculture brood stock is subject to genetic drift, a process that reduces genetic diversity over generations. Additionally, the source of the aquaculture brood stock is often unknown, and examples such as the unmanaged release and escape of aquaculture stock of Pacific salmon (*Oncorhynchus* spp.) have resulted in a loss of genetic variation or out breeding in wild populations (Reisenbichler and Rubin 1999). Therefore, surveys are needed at all potential release sites to reveal the presence of relictual populations to avoid compromising the long-term conservation of *A. davidianus* and other Cryptobranchids. Their population genetics must also be assessed to enable the provision of genetically competent individuals for release (Reisenbichler and Rubin 1999)

Consequently, the major conservation needs of *A. davidianus*, besides watershed restoration, limiting wild harvest, and pathogen management, are assessing the presence of relictual populations and their conservation genetics, and then matching the genetics of released stock with those found in nature. When these requirements are satisfied, the survey focus must include selecting suitable release sites, then release of juveniles or adults, and ongoing assessment of the survival and reproduction of released individuals. Because there are few remaining *A. davidianus* in nature, it will be difficult for surveys to associate habitat variables with carrying capacity (Zhang et al. 2002). However, surveys can identify remaining populations, provide genetic samples, and assess the success of restocking programs (Wang et al. 2004).

The conservation of *A. japonicus* relies on the maintenance of the populations that generally still remain in suitable habitats (Tochimoto et al. 2008). Although *A. japonicus* was harvested in the past, strict protection is now in place to prevent this species from exploitation. However, threats include habitat modification and other anthropogenic changes, including pollutants, and the introduction of *A. davidianus* in some systems. Consequently, the conservation needs of *A. japonicus* include surveying

Figure 2. Genetic drift and selection for color traits in *A. davidianus* have resulted in orange, piebald, and albino strains. *Image Robert Browne*.

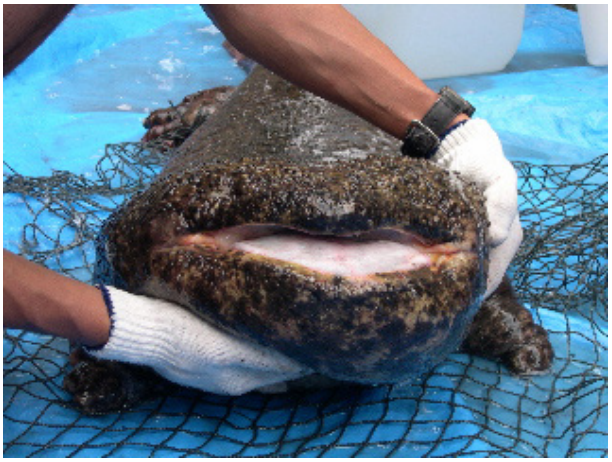

Figure 3. *Andrias japonicus* is the second largest Cryptobranchid and reaches 150 cm in total length and 44 kg in weight. *Image Sumio Okada*.

population densities and demography, habitat variables including channelization and watershed characteristics, assessing the effects of obstacle removal to migration, such as dams, and the provision of artificial habitats on survival and recruitment (Browne et al. 2012a, b).

The conservation needs of *C. alleganiensis* include identifying the most enigmatic threat to any Cryptobranchid and perhaps any amphibian species. *Cryptobranchus alleganiensis* has generally been declining over most of its range (Wheeler et al. 2003; Foster et al. 2009), to some extent due to habitat degradation and modification. However, *C. alleganiensis* still survives in near historic numbers in some locations, and some habitats modified by siltation and agricultural development still support substantial numbers of *C. alleganiensis*. However, the recruitment of *C. alleganiensis* has failed for decades over a substantial part of its range due to unknown causes, and many of these declining populations are now comprised of only a few old individuals (D. McGinnity, pers. comm.).

Cryptobranchus alleganiensis is subject to many ongoing surveys; however, these research activities have not revealed the cause of poor recruitment (Wheeler et al. 2003; Foster et al. 2009). Addressing this problem will require targeting the life history stage where the failure of recruitment occurs, from mating success through fertilization, to egg development, and larval and juvenile survivorship. Surveys will need to correlate recruitment to different life history stages with environmental variables such as pollutants. Attempts to reproduce *C. alleganiensis* in captivity for restocking are in the early stages of development, and no larvae have been produced. However, the production of large numbers of individuals from wild eggs has been successful and their release to natural habitats is underway. The cryopreservation of sperm is now being undertaken to perpetuate the genetic variation of populations with poor or no recruitment (National Geographic 2010; Michigan State University 2010). In addition, research has been initiated to provide a suite of reproduction technologies to produce genetically competent individuals (D. McGinnity, pers. comm.).

Cryptobranchids present particular survey challenges because of their large variation in body size, from three cm larvae to 1.5 m adults. Additional challenges include the wide variation in their aquatic habitats (deep turbulent water, shallow riffles, pools, lakes) and varied microhabitats (crevices, large rocks, pebble bed in riffles) (Nickerson and Krysko 2003; Tao et al. 2004; Okada et al. 2008). The habitats of *A. japonicus* and *C. alleganiensis* are relatively accessible, but, the habitat of *A. davidianus* includes difficult to survey, rugged, remote, fast-flowing interior rivers in the mountainous areas of central China (Tao et al. 2004).

Effective survey methods depend on associating the life stages of target species with their microhabitats. Adult Cryptobranchids live in cavities, under large rocks, and in bank-side dens. Because of the low population densities of the relictual populations of *A. davidianus*, recent surveys have relied on the observation of adults, electrofishing and the use of bow hooks (Wang et al. 2004). Surveys for adult and subadult *A. japonicus* in their habitats of slow flowing rivers have largely relied on direct observation with some netting (Okada et al. 2008). In contrast, surveys of adult and subadult *C. alleganiensis* have used a wide variety of techniques, including rock turning while snorkeling or, in deeper water, scuba diving or trapping (Nickerson and Krysko 2003; Foster et al. 2008). Recent innovations in survey techniques for *C. alleganiensis* include the use of artificial spawning sites to reveal reproductive success. The use of video cameras has the potential to increase observations of mating, brooding by males, and the development of oocytes and larvae. Environmental DNA (eDNA) detection (Goldberg et al. 2011) has the potential to both detect Cryptobranchids and to estimate their standing biomass and population. Radiotelemetry offers an opportunity to survey the movements and survival of an increasing size range of Cryptobranchids over an extended period (Kenward 2001).

Andrias japonicus and *C. alleganiensis* larvae and early juveniles are encountered less frequently than adults due to their particular microhabitats and to the low larval recruitment of *C. alleganiensis* in some regions (Nickerson and Krysko 2003; Okada et al. 2008). In contrast, the larvae of *A. davidianus* were commonly found in surveys of shallow mountain streams in the Qin Ling Mountains until their populations rapidly declined in the early 1980s (Zhang et al. 2002). Okada et al. (2008) found recently-hatched larvae of *A. japonicus* in pools under leaf litter or undercut banks, whereas more developed *A. japonicus* larvae were found under rocks and in gravel beds. Adults can be found in bunk burrows or among deeper rocks or branches. Although little is known about the microhabitat of the larval stages of *C. alleganiensis*, observations suggest that both larvae and small juveniles inhabit interstitial spaces under river gravel in riffles (Nickerson and

Krysko 2003; Foster et al. 2008). Juvenile and subadult *C. alleganiensis* most frequently occur in clean, rock-based streams, although they are also found in deeper pools with rocks, vegetation, and snags (Nickerson and Krysko 2003).

The efficacy of survey methods can vary through the interaction of climate and season with diel activity cycles. For example, the nocturnal activity of *C. alleganiensis* in streams of southeastern North America is positively correlated with high water levels (Humphries and Pauley 2000). Nocturnal surveys are most productive in late spring and early summer, whereas wire mesh baited traps were most efficient from early winter to late spring (J. Briggler, pers. comm.). Recent survey innovations for *C. alleganiensis* include the use of artificial breeding dens for adults, egg masses, and larvae, and the placement of natural rocks to provide habitat. Safeguarding the health and reproductive success of Cryptobranchids is critical when choosing survey techniques. Techniques necessitate minimal disturbance to the habitat, the use of sanitary procedures to prevent pathogen dissemination, and the protection of nest sites. If possible, several survey techniques should be used concurrently to improve survey accuracy and minimize sampling bias (Nickerson and Krysko 2003).

Survey design needs to incorporate the recognition of potential biases through the choice of technique, surveyed microhabitat, species, and life stage (Dodd 2009). Nowakowski and Maerz (2009) tested the efficacy of surveys of larval stream salamanders by comparing the mark-recapture success of passive leaf litter trapping and dip netting. Significant size bias occurred, with traps capturing a higher proportion of large individuals and dip netting yielding a greater proportion of smaller size classes. The survey efficiency of first and second order streams was greater at low salamander densities with time-constrained opportunistic sampling, but greater with quadrat sampling when salamanders were at high densities (Barr and Babbitt 2001). Nowakowski and Maerz (2009) concluded that the physical dynamics

Figure 4. *Cryptobranchus alleganiensis* has been the subject of the most diverse and innovative survey methods of all Cryptobranchids. *Image Dale McGinnity.*

Figure 5. Natural rock placed in stream to provide habitat and sampling locations for *C. alleganiensis*. *Image Kenneth Roblee.*

of water bodies and geographic region are primary considerations when selecting the most promising season for surveying different life stages.

An important consideration when surveying Cryptobranchids and other aquatic Caudata is the prevention and spread of infectious diseases. Chytridiomycosis (Chytrid; *Batrachochytrium dendrobatidis*) is an infectious disease of particular conservation concern for amphibians. Chytrid is an emerging pathogen that can regionally extirpate up to 90% of species and 95% of individuals in naive populations, at least among frogs (Lips et al. 2005). However, the effect of chytrid on Cryptobranchids has not been significant. One strain of chytrid has been suggested as endemic to populations of *A. japonicus* (Goka et al. 2009), and an undetermined strain of chytrid is found on mainland Asia in South Korea and may eventually impact *A. davidianus* (Yang et al. 2009).

Chytrid has been shown to be pathogenic in captive populations of *C. alleganiensis* (Briggler et al. 2007, 2008), although with apparently few, if any, pathological effects on natural populations. Nevertheless, good sanitation is a primary consideration in surveying Cryptobranchids, and other amphibians as a precaution against spreading chytrid. The same sanitary procedures will also prevent the spread of pathogens to other species of animals and plants. Another main pathogen currently threatening Cryptobranchids and other amphibians is *Ranavirus* (Geng et al. 2011). To prevent the spread of both amphibian chytrid and *Ranavirus*, equipment should be thoroughly sanitized when moving among aquatic systems, including all instruments, containers (e.g., measuring boards, weighing containers, and other instruments and equipment used), human body parts (hands), and clothing (especially, boots and waders) that come into contact with amphibians and their environment.

We review and compare the types and application of survey techniques for Cryptobranchids and other aquatic Caudata from a conservation and animal welfare perspective. Reviews or comparative studies of survey techniques for Cryptobranchids include Nickerson and

Krysko (2003; *C. alleganiensis*), Wang et al. (2004; *A. davidianus*), Okada et al. (2008, 2006; *A. japonicus*), and Dodd (2009) for general survey techniques of amphibians.

Survey techniques we review include: 1) *Wading, turning substrate, netting, and snorkeling*, 2) *Scuba/hookah diving*, 3) *Nocturnal spotlighting*, 4) *Bow-hooks/trot-lines*, 5) *Questionnaires*, 6) *Electrofishing*, 7) *Underwater camera systems*, 8) *Passive integrated transponders (PIT tags) and mark-recapture*, 9) *Radiotelemetry*, 10) *Modular artificial spawning dens and rock substrate placement*, 11) *Wire mesh baited traps*, 12) *Population genetic techniques*, and 13) *Environmental DNA (eDNA) detection*.

Review of survey techniques

1. Wading, turning substrate, netting, and snorkeling

Wading and turning substrate, coupled with snorkeling and downstream netting and seining, are widely used techniques for surveying *C. alleganiensis* and other Cryptobranchids (Taber et al. 1975; Peterson et al. 1983, 1988; Nickerson and Krysko 2003). These techniques are considered the most effective techniques in relatively clear shallow streams and pools less than one meter in depth with a substrate of rocks and other loose shelters (Nickerson and Krysko 2003). Cryptobranchids can be surveyed through blind searches by reaching beneath large rocks or within hollow logs or holes in banks. These techniques have resulted in the detection of hundreds to thousands of *C. alleganiensis* in some surveys (Taber et al., 1975; Peterson et al. 1983, 1988).

Snorkeling is another common technique for surveying *C. alleganiensis* (Nickerson and Krysko 2003) and other salamanders and is most effective in clear waters from 0.5 to < 3.0 m in depth. This method has proved more efficient than wading and turning substrate in surveys of *C. alleganiensis* in the gilled larval stage (Nickerson et al. 2002).

Foster et al. (2008) turned rocks to survey for adult and larval *C. alleganiensis* and captured 157 in 317 person hours (0.5 individuals per person hour (pph)). Bank searching through turning substrate within four meters of the stream bank yielded 14 juveniles in 55 person hours (0.25 pph). Bank searches of four of the seven inhabited sites yielded no *C. alleganiensis*, but at three sites bank searching was more efficient than rock turning (Foster et al. 2008). Capture rates of *C. alleganiensis* in four streams in the White River drainage, Missouri, varied from zero to 2.5 pph (Trauth et al. 1992). Okada et al. (2008) used diurnal wading and substrate surveys with one to three people searching under piled rocks or leaves (by hand or with dip-nets) to observe 227 *A. japonicus* at a rate of 1.4 pph.

Figure 6. Turning heavy rocks, combined with snorkeling with face masks and nets is an effective means to survey juvenile and adult *C. alleganiensis*. *Image Robert Browne.*

2. Scuba/hookah diving

Deep water habitats have not generally been well surveyed for Cryptobranchids, although standard scuba diving equipment and surface-based air compressor systems (hookah dive systems) are being used increasingly for surveying *C. alleganiensis* in fast-flowing, deep water two to nine meters in depth. Scuba diving allows for prolonged submergence giving the diver the capability to systematically check all available cover and often capture all individuals observed.

Standard scuba diving equipment provides unlimited mobility in terms of the area a worker can survey. In contrast, divers using a stationary anchored boat, canoe, or bank-side hookah system are limited by air line length.

Figure 7. Snorkeling and turning small substrate is a good technique for surveying small to large *C. alleganiensis* in water of moderate depth. *Image Robert Browne.*

Nevertheless, free-floating hookah systems are available that allow hookah divers to work in moderately fast waters with unlimited mobility as the compressor floats freely behind the divers. If conditions are not favorable for use of a free-floating hookah system, then a boat or canoe can be used to provide a semi-mobile platform for a stationary hookah compressor.

Boat-mounted hookah systems enable dives of one hour (hr) to more than 1.5 hr duration, and can be used at multiple sites during a full day of fieldwork without the need to refuel. Hookah systems require the use of a dive harness fitted with lead weight (usually 20-25 kg) sufficient to hold a diver in place in fast currents. The streamlined profile of hookah systems reduces the fatigue experienced by divers using standard scuba equipment. Divers also must be capable of working in fast moving water and have the physical strength to move large cover objects to successfully locate Cryptobranchids. For safety reasons, all diving requires a minimum of two divers, so that a "buddy system" is in place. If using a hookah dive system, a topside operator is required to monitor conditions and equipment. All divers must have appropriate certification and must surface when air cylinder pressure drops to 500 psi.

3. Nocturnal spotlighting

Nocturnal spotlighting has the advantage of producing minimal substrate disturbance, as rocks are lifted after the protruding heads of *C. alleganiensis* are observed. Spotlighting also allows observation of migratory and other behaviors. A spotlight survey of *C. alleganiensis* in West Virginia, USA, showed that increased nocturnal activity is correlated with high water levels, and suggested that spotlight surveys for mature adults are best conducted in May and June in this region (Humphries and Pauley 2000). Kawamichi and Ueda (1998) used nocturnal surveys combined with wading for *A. japonicus* in streambeds, and this technique, without substrate turning, is the most common survey technique for *A. japonicus*.

Figure 8. Artificial spawning dens for *C. alleganiensis* are used to increase the number of nesting sites and allow monitoring of egg production and larval survival. *Image Noelle Rayman.*

Nocturnal snorkeling/scuba surveys follow the same protocol as wading surveys, except that the observers are swimming and using dive lights to spot salamanders. Nocturnal snorkeling/scuba surveys have been conducted with some success in Missouri and Arkansas, USA, especially during the spawning period. Boats with halogen spotlights powered by generators have been used to survey for *C. alleganiensis* in Missouri (Wheeler 2007; Nickerson and Krysko 2003).

4. Bow-hooks/trot-lines

Bow-hooks or trot-lines can be an efficient survey technique in detecting the presence of Cryptobranchids at low population densities (Wang et al. 2004; Liu et al. 1991). Wild populations of *A. davidianus* have declined dramatically during the past 40 years, and in many regions bow-hooks may provide the most practical survey technique (Liu 1989; Wang 1996; Zhang and Wang 2000; Zhang et al. 2002).

Wang et al. (2004) surveyed *A. davidianus* using bow-hooks made of small pieces of bamboo fitted with four or five sharp hooks. In this study, only one *A. davidianus* was captured with the bow-hooks, whereas none were observed during night surveys and eight were captured by electrofishing. Bow-hooks were found to be an effective survey technique for *A. davidianus* in the remote and rugged Huping Mountain National Nature Reserve, an area of particular conservation significance (Zhang et al. 2002; Tao et al. 2004). Protection now forbids the use of hooks for surveying *A. japonicas*, although they can be captured without a hook by using bait on a stick (Tochimoto 2005). Bottom-set bank lines have been used in surveys of *C. alleganiensis* in sections of river with no rocks or logs, or that were unsuitable for wading and substrate turning (Dundee and Dundee 1965; Wortham 1970; Nickerson and Krysko 2003).

5. Questionnaires

Questionnaire surveys were conducted by Wang et al. (2004) with local fisheries managers and villagers to analyze the past and present distribution and status of *A. davidianus*. A total of 72 answered questionnaires concluded 1) *A. davidianus* were abundant prior to the 1980s, when individuals could be found easily and captured, 2) populations have since dramatically declined, and it is now difficult to capture *A. davidianus*, and 3) the main reasons for declines are excessive poaching, habitat fragmentation, and pollution. Responses to questionnaires also suggested that *A. davidianus* inhabited areas where 82 subsequent nocturnal surveys failed to detect them, so questionnaire results were neither verified nor discredited.

In another example of questionnaire survey, Tochimoto et al. (2008) collated data using questionnaires on the past distribution of *A. japonicus* in Hyogo Prefecture, western Honshu, Japan. A distribution map of *A. japonicus* was produced from the combined responses of oral interviews, answers to written questionnaires, and data from previous publications. Oral interviews were conducted with 17 people from fishermen's associations, two people from the nature conservation society in Hyogo Prefecture, and 21 people recommended by the fishermen's associations as very familiar with *A. japonicus*. The interviews were supported by information obtained through written questionnaires provided by the Boards of Education of 44 municipalities.

6. Electrofishing

Electrofishing requires a backpack voltage generator, connected to two submersible electrodes, carried by a researcher walking slowly through a stream. Amphibians and other aquatic vertebrates are first attracted to the electrical field of the electrodes and then temporarily paralyzed (Reynolds 1983).

Williams et al. (1981) considered electrofishing with seining effective for surveying *C. alleganiensis*. However, subsequent studies have not supported this conclusion (Bothner and Gottlieb 1991; Nickerson and Krysko 2003). In extensive river sections where large populations were found using other survey techniques, electrofishing failed to reveal *C. alleganiensis* (Nickerson and Krysko 2003). Electrofishing failed to locate *C. alleganiensis* during surveys on the Susquehanna drainage in New York, whereas turning rocks was successful (Soulé and Lindberg 1994). Substantial rock cover and poor water currents can result in shocked *C. alleganiensis* not moving from beneath rocks during electrofishing (Nickerson and Krysko 2003).

A two-year population study of another large aquatic salamander, the Common mudpuppy (*Necturus maculosus*), concluded that electrofishing was ineffective in surveying sites with large populations (Matson 1990). Nevertheless, there are examples of successful electrofishing for aquatic salamanders, especially when salamander abundance is being associated with other species abundance including fish. Maughan et al. (1976) used electrofishing to successfully survey the Pacific giant salamander (*Dicamptodon ensatus*), and Nakamoto (1998) exhaustively surveyed both fish and *D. ensatus* using multiple passes with backpack electrofishing. Occasionally, *C. alleganiensis* are incidentally captured with electrofishing by fisheries biologists during late summer/early autumn.

Because of its potential to harm salamander health and reproduction the use of electrofishing for surveys is not generally recommended, and should be confined to occupancy surveys of special conservation significance where other techniques are not effective. Electrofishing is well known for causing spinal injuries and mortality in fish (Cho et al. 2002; Wang et al. 2004), and there is potential for electric shock to reduce salamander reproductive success (particularly during the breeding season) and to damage the immune system (Nickerson and Krysko 2003). Electrofishing can seriously affect the health of critically endangered fish such as the Chuanshan taimen (*Hucho bleekeri*), and electrofishing is banned in the range of *H. bleekeri* in Taibai, Shannxi Province, China (W. Zhenghuan, pers. comm.)

Nevertheless, electrofishing may be the best technique for occupancy surveys in some difficult habitats where the detection of threatened salamanders is of major conservation significance (Nickerson and Krysko 2003). Wang et al. (2004) reported the capture of eight *A. davidianus* with electrofishing, whereas nocturnal surveys revealed none and bow-hooks only one (Zhang and Wang 2001).

7. Underwater camera systems

The use of waterproof video systems for surveys minimizes habitat disturbance, and video systems can locate den sites, record reproduction and behavior, and provide other valuable information on Cryptobranchid biology. Waterproof video systems are very effective where Cryptobranchids utilize heavy large rocks or bedrock crevices for shelter.

Black and white cameras have been used successfully. However, suitably small underwater color cameras are now available. Although color cameras are less light sensitive than black and white, the use of color is more efficient at revealing salamanders and eggs. We are not aware of an "off the shelf" video camera system optimal for surveying all Cryptobranchid species, or one that incorporates all features needed for efficient aquatic surveys. However, there are two relatively inexpensive systems available suitable for surveys of aquatic salamanders: 1) fishing video systems, and 2) inspection cameras.

Fishing video systems (12 volt) can easily be modified for surveys of Cryptobranchids. However the waterproof charged couple device (CCD) cameras associated with these systems are too large to access many crevices. These cameras are also relatively bulky and better suited to use from a small boat or canoe. Inspection cameras are very lightweight, and with small camera heads, have proven effective for surveying *C. alleganiensis*. A limitation of both systems is that standard monitors are relatively small and are not waterproof.

Video systems are being developed by researchers that are waterproof, lightweight, and incorporate a wireless camera system, digital recorder, and video goggles.

The video recorder, battery pack, and wireless components are placed inside waterproof bags and worn in a backpack. Improved waterproofing of video goggles and some components of wireless inspection cameras would provide greater flexibility in using these systems.

In addition to utilizing video camera systems for active surveying, cameras may be left in the field as a passive survey technique, if connected to a 12 V (volt) surveillance digital recorder. Batteries for the recorder need replacement, and data must be retrieved approximately once a week, depending on battery size and data storage capabilities of the recorder. Batteries are heavy and transport for recharging is arduous, but solar panels could be used to provide electricity in remote but secure locations.

8. Passive integrated transponders (PIT) and mark-recapture

PIT tags are small, waterproof, glass-encased capsules containing an alphanumeric code read with a portable reader. PIT tags are generally inserted sub-dermally with a syringe and needle, have life spans of at least 10 years, and are relatively inexpensive. PIT tags are available as read-only tags containing unique factory-set alphanumeric codes or as read-write tags that can be changed to any value. The new read/write PIT tags enable details to be recorded, retrieved or changed using the receiver, including the GPS location, habitat, tagger's name, and contact information. Gorsky et al. (2009) used 23 mm read/write PIT tags to assess Atlantic salmon (*Salmo salar*) migratory path selection. Although the size of PIT tags has steadily decreased, the detection range increases with PIT tag size. The standard reader ranges for read-only PIT tags are 3-8 cm for the smallest microchips (1.5 × 7 mm) and 15-45 cm for the largest (34 mm). Fish less than 55 mm have been successfully tagged using 11.5 mm PIT tags that weigh 0.1 g, and the smallest PIT tags now available should be suitable for all but the smallest Caudata.

A promising new technique, for surveying and locating salamanders in shallow water habitats is the use of submersible antennae and larger PIT tags that have been detected up to 90 cm through water (Hill et al. 2006) and detection range should further increase through improvements in antenna technology (Hamed et al. 2008). Cucherousset et al. (2008) showed that detecting Pyrenean brook salamanders (*Calotriton asper*) using PIT telemetry was 30% more efficient for individual sampling, and four times as efficient in sampling over time, than direct sampling through visual searching and rock turning. The efficiency of PIT telemetry was negatively correlated with the presence of large stones that blocked the PIT signal, and positively correlated with the number of easily sampled spring inlets and undercut banks (Cucherousset et al. 2008).

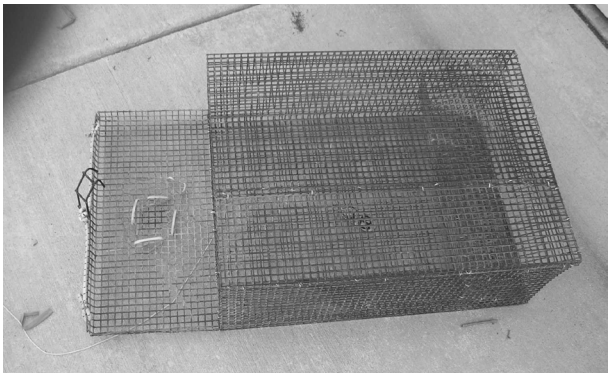

Figure 9. Trap used to capture *C. alleganiensis* in the Allegheny River drainage during the summers of 2004 and 2005. Bait (White sucker, *Catostomus commersonii*) was attached to the inside of the hinged door of a wire mesh cage. The bait cage was later removed and replaced using plastic zip ties. From Foster et al. 2008. *Used with permission from Herpetological Review.*

Bub et al. (2002) showed that when PIT tags were hidden within different stream microhabitats, more than 80% were subsequently located with portable antennas. Hill et al. (2006) tested specialized "PIT pack" antenna systems and found that design modifications and reduced equipment weight made PIT packs easy to use. The read range of optimized PIT packs approached 90 cm when the PIT tag was submerged in water. Breen et al. (2009) found a detection efficiency of 76% with PIT-tagged fish using a portable antenna investigating displacement, mean movement distance, and home range of Mottled sculpins (*Cottus bairdii*).

Prior to PIT tagging, photographs of head or tail spotting patterns were used to identify post metamorphic individual *A. japonicus* for mark-recapture studies (Kawamichi and Ueda 1998; Tochimoto 1991; Tochimoto et al. 2005). PIT tagging is the most common technique for mark-recapture studies. For example, Tochimoto et al. (2005) recorded 1204 individual salamanders in the Ichi River, Hyogo Prefecture, between 1975 and 2004, with 588 of these PIT tagged between 1998 and 2004. Okada (2006) tagged more than 500 individuals in Tottori Prefecture between 2001 and 2008.

Wheeler (2007) used the BioMark® submersible antenna with a detection distance of up to 30.5 cm to survey for previously PIT tagged *C. alleganiensis*. Of six *C. alleganiensis* marked using PIT tags, surveyors were able to detect only two the following day. A search of the area with rock turning did not detect any additional *C. alleganiensis*. The four undetected *C. alleganiensis* had either moved into water deeper than the reach of the detector wand antenna (two meters) or moved under the cobble substrate (Wheeler 2007).

Automatic systems to survey movement have been used with PIT tags in fisheries research. These consist of remote antenna arrays spanning water bodies. Meynecke et al. (2008) successfully used remote PIT technology to monitor fish movement for 104 days in a mangrove

Table 1. The advantages and disadvantages of survey techniques.

Survey technique	Advantages	Disadvantages
1. Wading, turning substrate, netting, and snorkeling.	Low equipment costs. Simple and rapid surveying. Snorkeling provides better vision and a closer proximity to exposed *C. alleganiensis*. Rocks can be tilted more easily due to buoyancy and water currents can provide "lift" of rocks.	Cannot sample deep water, surveyor strain and fatigue are high, and there is considerable habitat disturbance. Risks of blind searches include bites and cuts and rock turning can result in being held under water by a trapped arm. Some institutions will not allow surveying alone due to risk of injury. Costs for wetsuits, mask, snorkel, dive boots, and other equipment. Transporting heavy equipment (along shallow mountain streams) and working in high velocity areas can produce increased surveyor strain and fatigue.
2. Scuba/hookah diving	Deeper water habitats can be surveyed that are not accessible to other methods besides traps and trot-lines. Diving enables prolonged submergence, with less fatigue than snorkeling, at depths of one to two meters. Systematic checking of all cover and ensuring the capture of all exposed Caudata.	Surveying multiple sites requires the transport and handling of many air cylinders. Refilling air cylinders when at remote survey sites requires extensive transportation time. Requires substantial equipment costs including scuba or hookah equipment and sometimes boats, and extensive training time and costs. Diving is more dangerous than other surveying methods. It is time consuming to sanitize snorkeling, scuba and hookah diving equipment.
3.Nocturnal spotlighting	Nocturnal lighting creates little habitat disturbance, and enables the simultaneous survey of other nocturnal amphibians.	Potential costs of equipment (lights and boats), limited visibility through poor water clarity, and increased safety concerns.
4. Bow-hooks/trot-lines	Efficient for the detecting of the presence/absence and population assessment of Cryptobranchids at low population densities.	Bow-hooks (using fishing hooks) can cause injuries to salamanders, increase salamander stress over hand collecting, and increase predation risk. Bow-hook lines should be made too short to reach the esophagus and possibly cause injuries.
5. Questionnaires	Regional assessment of occupancy.	Relies on credibility of respondents.
6. Electrofishing	Presence/absence and population surveys in difficult habitats of major conservation significance.	Electrofishing for surveys is not generally recommended because of its potential to harm salamander health and reproduction and its use should be confined to occupancy surveys of special conservation significance where other techniques are not effective. Electrofishing has high equipment costs, a number of particular safety concerns, and requires several surveyors working together.
7. Underwater camera systems	Minimal habitat disturbance, location of den sites, recording of reproduction and behavior, and provision of other information on Cryptobranchid biology. Video camera systems can provide a passive survey technique in combination with a digital recorder.	Problems with waterproofing, battery charging and supply, limited water depth, and viewing monitors in bright sunlight. Costs can be high with this method for camera, recorder, and monitor, and only a single site can be monitored per camera.
8. Passive integrated transponders (PIT) and mark recapture	Recorded information can be retrieved from tagged salamanders (with limited habitat disturbance) enabling calculation of movement and dispersal. Allows tracking of confiscated animals.	Only previously tagged animals are detectable, a relatively short detection range, the workable water depth being limited by wand length, and detection range limited by shelter type and depth. PIT tag surveys using hand readers are economical; however, optimized antenna systems are costly. PIT tags can be lost.
9. Radiotelemetry	Monitoring of individuals to study movements, habitat use, and survival. Smaller, lighter, longer-lived, and more reliable units have increased the efficacy of radio-tracking with increasingly smaller individuals.	Surveys can be costly due to the initial expense of transmitters, antennas and receiver. Surgical implant is required for attaching transmitters to salamanders.
10. Modular artificial spawning dens and rock placement	Modular artificial spawning dens provide efficient means to support critical spawning habitat, enable monitoring of egg and larval survival, and survey male and female occupancy and movement. Further development of the capacity to provide camera surveillance will increase all the above.	Modular artificial spawning dens are relatively easy to construct but there are material and labor costs. They are heavy and require vehicular transport and a team to place in selected locations. Their stability under exceptionally high stream velocities, in comparison to natural rock dens, is untested.
11. Wire mesh baited traps	Trap surveying is not hampered by deep, turbid, or cold water. There are low levels of habitat disturbance, and sites with very heavy rocks and ledges can be surveyed.	Material and labor costs for trap construction, and supplying a large amount of fresh bait. Setting traps is labor intensive and transporting traps to remote areas may be prohibitive. Trapping should not be performed during the breeding season because females may spawn in the traps, and trapped males cannot guard dens. Flooding may carry away traps. Lost traps may be a hazard to wildlife. As with all unguarded equipment, theft or vandalism may be a problem.
12. Population genetic techniques	Minor tissue sampling enables ongoing studies of the number and significance of genetic subpopulations, loss of genetic variation, migration and dispersal, effective population size, and parentage. Samples can be subdivided and provide material indefinitely for future work and comparison.	Contamination and poor storage of samples limits analysis. Cryptobranchids and some other Caudata have low genetic variation, which can limit the use of techniques. More sophisticated genetic techniques are expensive.
13. Environmental DNA (eDNA) detection	Inexpensive, no habitat disturbance, can be used in streams difficult to monitor by other methods, shows occupancy.	Targeted primers need to be designed to amplify a species-specific short DNA fragment. Laboratory costs per sample and the need for several samples to exclude false positives or negatives. Efficiency depends on DNA shedding rates, population demography, water temperature, and thermal properties, to estimate population size.

stream and recorded more than 5000 detections with a recapture rate of 40%. River monitoring systems for fish commonly use four different types of antennas: pass-through, flat plate, crump weir, and circular culvert antennas. Flat plate detectors appear ideal for salamanders as they can be up to six meters in size, are buried slightly in the streambed, and can detect salamanders up to 45 cm above the plate.

The problem of PIT tag loss can be substantially reduced by careful application and sealing of the insertion site (Christy 1996). A coincidental value of PIT tagging to conservation is that resource managers and international border inspectors can utilize PIT tags to identify home locations of confiscated salamanders.

9. Radiotelemetry

Radiotelemetry can consistently be used to monitor individual animals and has been used to study movements, habitat use, and survival of many vertebrate species (Kenward 2001). Radio transmission can be received in turbid waters, stream flows, or depths that preclude traditional survey techniques (e.g., rock turning and visual searches). Surveys using radio-telemetry with *C. alleganiensis* have investigated dispersal (Gates et al. 1985b), site fidelity, and frequency and timing of movements (Coatney 1982; Blais 1996; Ball 2001). These surveys have revealed the use of unique microhabitats including bedrock ledges, root masses, and bank crevices (Blais 1996) as well as the location of den sites and causes of mortality (C. Bodinof, pers. comm.).

Monitoring by radiotelemetry requires attachment of a very high frequency (VHF) radio transmitter to the target salamander. Each transmitter is tuned to a unique frequency and emits a pulsed radio signal allowing an observer to locate individual salamanders. Optional sensors to detect motion, pressure, depth, or temperature can be incorporated into radio transmitters. To extend battery life, microcontrollers have been developed to turn transmitters on and off at preset times (Rodgers 2001). Technological advances have resulted in smaller, lighter, longer-lived, and more reliable units. Such advances have increased the efficacy of radio-tracking in increasingly smaller organisms while minimizing concern for adverse effects of transmitter attachment.

Several methods of transmitter attachment have been used with varying success for Cryptobranchids, including 1) coelomic implant (Blais 1996), 2) subcutaneous implant (Blais 1996), 3) force-feeding (J. Briggler, pers. comm.), 4) neck collar (Wheeler 2007), and 5) suturing through the tail (Okada et al. 2006; Wheeler 2007; Blais 1996).

Wheeler (2007) observed poor retention with external tail attachments, as well as collars fastened around the neck of *C. alleganiensis*. However, Okada et al. (2006) reported that transmitters attached externally (sutured through the tail) to large *A. japonicus* were retained for two to four months and caused minimal injuries. Radio transmitters were force fed and retained for 18 to 30 days (Coatney 1982), and 16 to 25 days (Blais 1996), in *C. alleganiensis* with no harm. Force-feeding transmitters may be useful for detecting untagged Cryptobranchids, which aggregate during a relatively short breeding season. Surgical implantation of transmitters should be performed by an experienced veterinarian or biologist (Fuller et al. 2005), and amphibians should be given ample recovery time from effects of anesthesia and surgery before release (Byram and Nickerson 2008).

A recommendation to minimize the effect of transmitter attachment is the use of the smallest possible tag. Transmitters also should not exceed 3-5% body mass and researchers should use the least conspicuous attachment technique (Withey et al. 2001). Jehle and Arntzen (2000) used very small transmitters of 0.5 g to track individual *Triturus* spp. above a minimum acceptable body mass of 8.0 g. PIT tag tracking may be useful for salamanders smaller than 8.0 g, but radio tracking antenna systems are cheaper, and radio tracking has a much greater range than PIT tags. Different sizes, battery life, outputs, and ranges of these and various other transmitter models have been used for radio-tracking Caudata. While trade-offs exist among unit weight, detection range, and battery life, many small units offer ≥ six months of battery life. Resources providing an overview of radio-tracking technology and study design include Fuller et al. (2005), Millspaugh and Marzluff (2001), and White and Garrott (1990).

Radiotelemetry studies of Caudata include *T. cristatus*, *T. marmoratus* (Jehle and Arntzen 2000), *Ambystoma maculatum* (Madison 1997; Faccio 2003), *A. jeffersonianum* (Faccio 2003), *A. californiense* (Trenham 2001), *C. a. alleganiensis* (Gates et al. 1985a; Blais 1996; Ball 2001), *C. a. bishopi* (Coatney 1982), and *A. japonicus* (Okada et al. 2006).

10. Modular artificial spawning dens and rock substrate placement

A recent innovation in survey techniques for Cryptobranchids is development of modular artificial spawning dens. Bankside artificial dens have been used for *A. japonicus* in channelized habitat (where suitable sites were lacking), and in artificial streams for reproduction during farming of *A. davidianus*. The Ozark Hellbender Working Group developed modular spawning dens for *C. alleganiensis* that proved highly successful in attracting *C. alleganiensis* and providing spawning sites. Dens made of ferrocement are light, simple, and economical to construct. Artificial dens offer the possibility of incorporating underwater video systems giving discrete and continuous monitoring of occupancy and activity. Rocks

have been placed in streams to similarly provide habitat and increase survey efficiency for *C. alleganiensis*.

11. Wire mesh baited traps

Cryptobranchus alleganiensis have been surveyed over several years using baited traps in deep water habitat of some larger (7th order) rivers (including the Gasconade River, Missouri, USA). Such habitats have proved difficult to survey without trapping due to their depth (> 5 m maximum) and often very turbid waters (lateral Secchi Disk < 1.0 meters visibility). The efficiency of baited traps varies with water temperature (Nickerson 1980); trapped *C. alleganiensis* in deep rivers in Missouri were greatest during the peak foraging period in spring and very low during the summer breeding season. When water temperatures reached above 22 °C, capture rates were very low. Besides seasonal effects, trapping is highly dependent on how the trap is set. Foster et al. (2008) had greatest success when bait was fresh and the trap was flush with the substrate.

Wire mesh baited traps have been widely used to survey Cryptobranchids using a variety of baits. *Cryptobranchus alleganiensis* can detect baits from considerable distances (Townsend 1882; Nickerson and Mays 1973), and smelly, fresh baits are most successful in trapping. Traps baited with chicken livers proved unsuccessful with *C. alleganiensis* (Soulé and Lindberg 1994). Foster et al. (2008) used similar traps successfully when baited each day with fresh fish; fresh meat bait proved unsuccessful. Kern (1984) successfully captured *C. alleganiensis* using hoop-nets baited with fresh sucker fish (*Carpiodes* sp.). Trapping with crab traps baited with strong smelling saltwater baits (such as sardine, mackerel, or squid) was effective for catching adult *A. japonicas* (S. Okada, pers. comm.). When surveying Cryptobranchids, the bait bags should be strong enough to resist tearing from salamander bites and the possible ingestion of bag material. Trapping should not be performed during the breeding season because females may spawn in the traps, and trapping can prevent males from guarding nests.

The Missouri Department of Conservation, USA, has a major survey program for *C. a. alleganiensis* using traps in habitats unsuitable for other methods. Trap design was modified from those used by Foster et al. (2008; Figure 8) by placing a funnel on both ends and making the traps collapsible to reduce storage space. Numerous bait types (chicken liver, crayfish, carp, and Gizzard shad) were used as bait, but fresh Gizzard shad (*Dorosoma cepedianum*) was the most successful bait. Besides the bait used, the general success of trapping is also highly dependent upon how the trap is set.

Trapping is a valuable sampling technique used for *C. alleganiensis*. In a comparative study, Foster et al. (2008) reported on three techniques of surveying Hellbenders: rock turning, bank searches, and trapping. Rock turning had the highest capture efficiency but damaged the habitat; bank searches were effective at finding juveniles. Besides its use in habitat accessible to other techniques, trapping was useful for water slightly exceeding the maximum depth possible with other techniques and in areas with unmovable rocks or difficult-to-access ledges. Trapping may be more effective for capturing the largest size classes (Figure 10; Foster et al. 2008). Trapping is similarly effective for catching adult *A. japonicus* (S. Okada, pers. comm.). Snorkeling, scuba, or hookah diving combined with trapping would enable better trap placement, especially at greater depths.

12. Population genetic techniques

Genetic information can guide conservation breeding programs determining the number and significance of genetic subpopulations. Using increasingly sophisticated genetic techniques, evolutionary phylogeny, paleogeography, species status, migration, effective population size, parentage, and population bottlenecking can be ascertained. Surveys using molecular techniques to assess population genetic structure, variation, and migration patterns have rapidly progressed over the last 10 years. This progress has been largely driven by improved sequencing and computer analysis, Information Technology systems, and a growing bank of genetic techniques and resources (GenBank Database 2009).

Mitochondrial techniques are useful for understanding relationships among and historical changes within populations (Sabatino and Routman 2009), however, mitochondria are maternally inherited and only track female lineage.

Genomic microsatellite markers, together with mitochondrial DNA information, may provide the most informative phylogenetic information. Microsatellite markers have the advantage of requiring very little tissue (even less than used in mitochondrial sequencing techniques) and this allows for noninvasive sampling such as buccal swabs. Polymorphic microsatellite markers have very recently been published for *C. a. bishopi* (Johnson et al. 2009) and *C. a. alleganiensis* (Unger et al. 2010).

13. Environmental DNA (eDNA) detection

Environmental DNA (eDNA) has recently been confirmed as a sensitive and efficient tool for inventorying aquatic vertebrates in lotic and lentic aquatic habitats. Under the Amphibian Research and Monitoring Initiative, U.S. Geological Survey scientists and their partners developed an efficient protocol for detecting eDNA from two amphibian species that occur in low density, fast-moving stream water; the Idaho giant salamander (*Dicamptodon aterrimus*) and the Rocky Mountain tailed

frog (*Ascaphus montanus*). Environmental DNA analysis costs approximately US$30. Sampling efficiency increases in comparison with fieldwork, for example, by 20 times for *D. aterrimus* and 11 times for *A. montanus* (direct survey population estimates of 0.16 and 0.04 individuals per m^2, respectively). With Asian carp, sampling cost efficiencies increase from 16 to 100 times when compared to field searches. The sensitivity of an eDNA test depends on the sampling of five to 10 litres of water, the amount of DNA shed by the target species, and the thermal and chemical properties of the water. False negative rates can be estimated using repeated sampling, and the probability of false positives can be excluded by careful primer design and protocol testing using related non-target species (Goldberg et al. 2011).

Conclusion

Cryptobranchids are iconic amphibians that provide a range of conservation challenges. Of all the aquatic amphibians, Cryptobranchids appear to offer the greatest potential to link amphibian conservation with watershed management. They also offer the greatest potential to apply a suite of modern and innovative techniques to conservation strategies. Their long-term survival is highly dependent on the effectiveness of these survey techniques to elucidate population structure and demography, bottlenecks in recruitment, threats, and critical habitat components.

There is a wide variety of survey techniques to detect, capture, and track Cryptobranchids and other aquatic Caudata. However, these techniques vary widely in efficacy, and a combination of several techniques will prove most effective at providing critical information on occupancy and status. Each survey technique has advantages, disadvantages, and biases depending on survey objectives (Nickerson and Krysko 2003).

When choosing survey techniques, a primary concern is animal welfare. The preservation of nest sites and other critical habitat is essential, as is limiting the spread of pathogens. Suitable *C. alleganiensis* nesting sites are increasingly scarce in many locations, and in some locations siltation is destroying the sites that remain. Underwater camera systems are the only survey techniques that do not disturb habitat, especially when used with artificial spawning dens. Only radiotelemetry, PIT tagging with long-range detection, and environmental DNA (eDNA) detection enable ongoing sampling without further habitat disturbance (Nickerson and Krysko 2003).

Wading shallow water and turning substrate, including leaves and gravel, is a simple way to survey Cryptobranchids and may be efficiently combined with surveys of larvae and juveniles. Survey efficiency for adult and larval Cryptobranchids, and other Caudata through rock turning, is improved by the use of downstream seines. Scuba or hookah diving are the only techniques that detect all sizes of gilled larvae and multiple age groups of non-gilled and adult Cryptobranchids within short survey periods, but they are one of the most expensive and training-intensive methods. The use of eDNA promises the most rapid and cost effective survey technique for the inventory of Caudata.

Final remarks: Cryptobranchids are one of the most endangered groups of Caudata, having highly specialized habitat requirements at different life stages. Various sur-

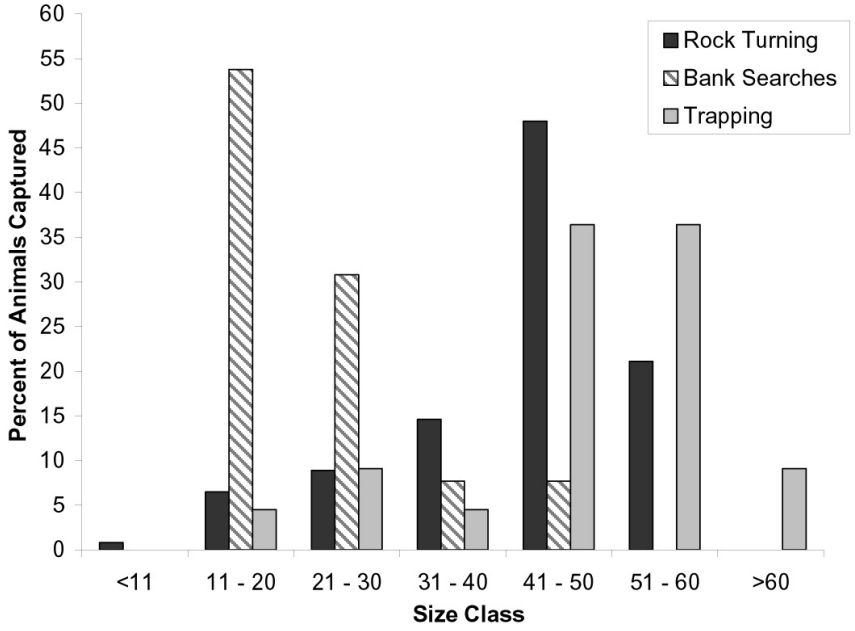

Figure 10. The relative success of three capture techniques in locating various size classes of *C. alleganiensis*. From Foster et al. 2008. *Used with permission from Herpetological Review.*

vey techniques offer a range of advantages and disadvantages, and surveys should include several techniques to reduce bias. Cryptobranchids' high site fidelity and reliance on easily damaged critical habitat components make them vulnerable to survey techniques that require disturbing habitat structure. Therefore, the choice of survey technique should always include minimum habitat disturbance and potential to affect salamander health. Equipment must be sanitized when moving among sites to limit the spread of pathogens.

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The giant salamanders (Cryptobranchidae): Part A. palaeontology, phylogeny, genetics, and morphology

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***Abstract.*—The Cryptobranchidae, commonly called the Giant Salamanders, are the largest surviving amphibians and comprise two extant genera, *Andrias* and *Cryptobranchus*. There are three cryptobranchid species, the Chinese giant salamander (*Andrias davidianus*; 180 cm, 59 kg), the Japanese giant salamander (*A. japonicus*; 155 cm, 55 kg), and the North American giant salamander (*Cryptobranchus alleganiensis*; 74 cm, 5.1 kg). Because of their iconic status as the world's largest amphibians and their biopolitical significance, all cryptobranchids are subject to major and expanding initiatives for their sustainable management. Cryptobranchids are biologically similar in many ways; however, within these similarities there are differences in their habitats, diet, size, reproductive behavior and seasonality, fecundity and egg size, paternity, and growth and development. These characteristics are a consequence of their palaeontology, phylogeny, genetics, and morphology. Cryptobranchid conservation genetics reveal the evolutionary significant units (ESUs) toward which conservation and research efforts must be directed to provide genetically competent individuals for rehabitation or supplementation programs. Knowledge of these scientific fields in concert with cultural, political, and economic factors all contribute to cryptobranchid conservation biology and the formulation of optimal strategies for their sustainable management. However, there has previously been no comparative review of the numerous scientific fields contributing to the knowledge of cryptobranchids, and little peer-reviewed material on *A. davidianus* and *A. japonicus* has been published in English. Here we present the first article in a series about cryptobranchid salamanders, "The giant salamanders (Cryptobranchidae): Part A. paleontology, phylogeny, genetics, and morphology."**

Key words. Giant salamander, cryptobranchid, palaeontology, phylogeny, genetics, morphology, conservation, sustainable management, Cryptobranchidae

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Introduction

"The giant salamanders (Cryptobranchidae): Part A. palaeontology, phylogeny, genetics, and morphology" is the first of a series of three review articles that have been produced to review the biology and sustainable management of giant salamanders. Although there has been much published on giant salamanders, the information has previously been scattered within articles on each of the three species largely in languages of their biopolitical regions: Mandarin Chinese, Japanese, and English.

To maximize the potential for the sustainable management of these species, the public and scientific community must have access to accurate knowledge about them to direct policy and provide for Internet-based information and news portals. Consequently, "The Giant Salamanders (Cryptobranchidae)" suite of articles, review and discuss a broad range of biological data known for giant salamanders, which have been collected globally by researchers and enthusiasts over a period of four years.

Different authors have made varying contributions to each article depending on their area of expertise. However, due to the complexity of rewriting and contributing to

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Figure 1. A North American giant salamander (*Cryptobranchus alleganiensis*) shows the characteristic morphology of the cryptobranchids; large robust dorso-ventrally flattened head and body, small eyes, thick legs with stubby digits, lateral folds of skin for respiration, and sensory papillae for detecting water movement and prey (laterally flattened tail not shown). *Image and copyright by Ray Miebaum.*

the suite of articles as it has progressed over many years, we have included all authors on all articles. The major contributing authors to "The giant salamanders (Cryptobranchidae): Part A. palaeontology, phylogeny, genetics, and morphology" are Amy McMillan and Paul Hime (genetics), Raul Diaz (palaeontology, genetics), and Paul Hime (phylogeny).

The caudate superfamily, Crytobranchoidea is one of the most ancient amphibian clades and comprises two families Cryptobranchidae and Hynobiidae, totalling 51 species. The family Cryptobranchidae derives its name from the Ancient Greek, "kryptos" (hidden) and "branchos" (gill), which originally referred to the gills which must be hidden in adults as they lack external gills, unlike most aquatic vertebrates (larvae have external gills). The Cryptobranchidae, or "Giant Salamanders," are the largest surviving amphibians and comprise two genera, *Andrias* and *Cryptobranchus*. There are only three extant cryptobranchid species, the Critically Endangered, Chinese giant salamander (*Andrias davidianus* Blanchard, 1871), the Near Threatened, Japanese giant salamander (*A. japonicus* Temminck, 1936), and the North American giant salamander (*Cryptobranchus alleganiensis* Daudin, 1803) which exists as two formally named subspecies, *C. a. alleganiensis* and *C. a. bishopi* (Petranka1998).

The Crytobranchoidea, along with probably (Larson 2003) the fully aquatic caudate family Sirenidae are exceptional within the Caudata (salamanders) in having the reproductive mode of external fertilization (Duellman and Trueb 1994). As giant salamanders are the largest amphibians in their respective major biopolitical regions, they are conservation icons, not only for threatened amphibians but also, for the sustainable management of watersheds. Sustainable management requires providing the broadest range of educational material that relates to both public interest and species conservation. This knowledge can then be used by field, conservation breeding, and culturally engaged conservationists, to provide the best technical approaches to species conservation, and provide a background for the required political and financial support.

A critical part of this knowledge is the paleontological history and phylogeny to show a species' evolutionary significance, and how a species fits into the tree of life; while conservation genetics shows its evolutionary significant units (ESUs) for directing conservation and research efforts. However, there has been no comparative review of the conservation biology of cryptobranchids and associated scientific fields, and little peer-reviewed information of the conservation biology of *A. davidianus* and *A. japonicus* has been published in English.

Here we review "The giant salamanders (Cryptobranchidae): Part A. paleontology, phylogeny, genetics, and morphology" in concert with "The giant salamanders (Cryptobranchidae): Part B. range and distribution, demography and growth, population density and size, habitat, territoriality and migration, diet, predation, and reproduction" and "The giant salamanders (Cryptobran-

Figure 2. Fossil salamanders strongly support an east Asian (red ellipse) origin for the Cryptobranchoidea. The continents were distributed very differently in the Mid-Jurassic (170 MYA) before continental drift moved them to their present locations. However, Eurasia and North America remained in the Northern Hemisphere. By the Late Pliocene (3 MYA) the continents had moved to their present positions. *Image courtesy of palaeos site: http://palaeos.com/mesozoic/jurassic/midjura.html. Adapted from Gao and Shubin, 2003.*

chidae): Part C. etymology, cultural significance, conservation status, threats, sustainable management, reproduction technologies, aquaculture and conservation breeding programs, and rehabitation and supplementation."

Palaeontology and phylogeny

The Cryptobranchoidea is comprised of the giant salamanders, family Cryptobranchidae (found in China, Japan, and eastern North America), and the Asiatic salamanders, family Hynobiidae (found throughout Asia and European Russia). From fossil evidence in Asia, the evolutionary origins of the Cryptobranchidae extend to at least the Mid-Jurassic (160 million years ago [MYA]; Gao and Shubin 2003), with their fossils later being known from Europe, Asia, and North America. Fossils of more recent cryptobranchids from the Late Eocene (40 MYA) to the Early Pliocene (5.3 to 3.6 MYA) are known from two genera and two or three species from over 30 Eurasian localities (Böhme and Ilg 2003). Molecular and morphological studies strongly suggest an Asian origin for cryptobranchids with subsequent expansions into Europe and North America by the Upper Paleocene (3.6 to 2.5 MYA). The expansion into North America was probably facilitated by the resumption of ice ages creating a land bridge between Asia and North America during the Late Pliocene-Early Quaternary glaciation that started about 2.6 million years ago (Kruger 2008).

This basal caudate salamander family has experienced remarkable morphological stasis throughout its evolution, with ancient and modern Cryptobranchids being morphologically very similar. The Late Oligocene (23.0 MYA) to Early Pliocene (5.3 MYA) species *A. scheuchzeri* was distributed from Central Europe to the Zaissan Basin on the border of Kazakhstan and China. Vasilyan et al. (2010) considered from fossil and paleoclimatological evidence that both fossil and extant *Andrias* species occur in regions with annual precipitation from 90 to 130 cm.

The monophyly of the Cryptobranchoidea (Hynobiidae + Cryptobranchidae) has not been a point of contention (Gao and Shubin 2003; Larson and Dimmick 1993; Larson et al. 2003; Frost et al. 2006; Roelants et al. 2007; Pyron and Wiens 2011), though the base of the salamander phylogeny, relative to the placement of widely accepted clades, has been contentious for many decades, specifically due to the placement of Sirenidae and the relationship of other paedomorphic taxa (see: Wiens et al. 2005; Vieites et al. 2009). Salamanders have displayed a relatively conserved tetrapod body plan, at least since the Jurassic Period (Vieites et al. 2009). The independently derived paedomorphic morphology (a heterochronic change where sexually mature adults retain several aspects of the larval body plan) displayed by several recognized families, has played a central role in discussions of salamander morphology, and whose morphological characters have been considered to play a substantial confounding role in phylogenetic reconstruction.

Fossil cryptobranchids from the Late Eocene to the Early Pliocene are known from two genera and two or three species from over 30 Eurasian localities (Böhme and Ilg 2003; Milner 2000). Phylogenetic and paleontological evidence suggests an East Asian origin for cryptobranchids by, at latest, the Cretaceous (135-100 MYA),

Figure 3. The Late Oligocene to Early Pliocene (23.0 to 5.3 MYA) species *A. scheuchzeri* was distributed from Central Europe to the Zaissan Basin on the border of Kazakhstan and China. Fossil room II, Teylers Museum, The Netherlands *Andrias scheuchzeri* Oeningen. *Courtesy of: http://en.wikipedia.org/wiki/Andrias_scheuchzeri*

with subsequent expansions into Europe and North America by the Upper Paleocene (Milner 2000) via the Asian-American interchange (Duellman and Trueb 1994), though an alternate scenario has been proposed but not widely accepted (Naylor 1981). This basal caudate family has experienced remarkable morphological stasis throughout its evolution, with ancient and modern cryptobranchids appearing very similar, and neoteny being present since the time of early salamander origins (Gao and Shubin 2001; Gao and Shubin 2003). *Andrias* are morphologically conservative and their skeletons are so similar that *A. davidianus* has been considered a junior synonym of *A. scheuchzeri* (Westphal 1958).

Currently recognized fossil cryptobranchids include *Chunerpeton tianyiensis* (Gao and Shubin 2003), the earliest crown-group member, *Cryptobranchus* (=*Andrias*?) *saskatchewanensis* (Naylor 1981), and *Piceoerpeton willwoodensis* (Meszoely 1967; described from a single vertebra). *Cryptobranchus guildayi* (Holman 1977) was also described, based on limited samples and whose validity had previously been questioned (Estes 1981; Nickerson 2003), but whose apomorphies have recently been dismissed due to as yet undescribed intraspecific skeletal variation for *C. alleganiensis*, and the misidentification of the ceratohyal, which was actually a sacral rib; this taxon is thus synonymous with *C. alleganiensis* (Bredehoeft 2010). *Andrias matthewi* has also been described from Nebraska from a single mandible (Cook 1917; see Estes and Tihen 1964; and Naylor 1981). *Zaissanurus beliajevae* has been described from the Eocene/Oligocene of Mongolia and Russia while *Aviturus exsecratus* and *Ulanurus fractus* have been described from the Paleocene of Mongolia (Gubin 1991; Milner 2000).

Cryptobranchoid salamanders (Hynobiidae + Cryptobranchidae) share several synapomorphies including: high chromosomal counts (Hynobiidae: 2*n* [diploid number] = 40-78 and Cryptobranchidae: 2*n* = 60); extremely large nuclear genomes (Hynobiidae: 15.2-46.5 Gbp [Giga base pairs] and Cryptobranchidae: 45.5-53.8 Gbp) (Gregory 2012. Animal Genome Size Database. http://www.genomesize.com [Accessed: 12 June 2012]); presence of a hypoglossal foramen and nerve (Fox 1957; Fox 1959); fusion of the first hypobranchial and first ceratobranchial into a single structure, as well as the fusion of the *M. pubotibialis* and *M. puboischiotibialis* (Duellman and Trueb 1994); and retention of a separate angular bone in the lower jaw (Fox 1954; Fox 1959; Zhang et al. 2006; Vieites et al. 2009). Members of the Cryptobranchoidea display other primitive features such as external fertilization (also present in Sirenidae) and the production of eggs either as paired clusters (hynobiids) or strings (cryptobranchids), with one set from each oviduct (Duellman

and Trueb 1994). Cryptobranchid salamanders are specialized for an aquatic habitat of cold, fast flowing, rocky, and oxygen rich streams (Petranka 2010).

Extensive epidermal folds (with a dense subsurface capillary network) are present along the flanks of the trunk and limbs to increase surface area, serving as a body length "gill" for oxygen exchange, with the lungs thought to function only for buoyancy (Guimond and Hutchison 1973). Larval cryptobranchids have a dorsal tail fin and short external gills as do the majority of transforming salamanders. Adult *Cryptobranchus* maintain a single pair of gill clefts, while all are closed in *Andrias* (Duellman and Trueb 1994; Dunn 1922; Meszoely 1966; Rose 2003). The development of an angular bone and lack of a septomaxilla, lacrimal, and os thyroideum are shared skeletal characters of cryptobranchids (Fox 1954, 1959; Rose 2003), while diagnostic generic differences are the presence of four bones contributing to the border of the naris in *Cryptobranchus* (premaxilla, maxilla, nasal, and frontal), with a lack of the frontal bone contacting the naris in *Andrias* (Dunn 1922; Meszoely 1966). *Cryptobranchus* also fails to resorb the third and fourth ceratobranchials (Rose 2003). Other skeletal and ontogenetic differences can be found in Rose (2003).

Cryptobranchoidea, from genetic inference, are considered to have evolved during the Middle to Late Jurassic (Gao and Shubin 2003; Roelants et al. 2007; San Mauro et al. 2005; Zhang et al. 2005; Mueller 2006; Wiens 2007; Zhang and Wake 2009), while some researchers estimate early Cretaceous (Marjanovic and Laurin 2007; San Mauro 2010). Mitochondrial and nuclear DNA analysis shows the family Cryptobranchidae is a monophyletic group (e.g., Weisrock et al. 2005; Matsui et al. 2008; Zhang and Wake 2009) and that the two genera within this family, *Cryptobranchus* (North America) and *Andrias* (Asia) diverged between the Late Cretaceous to the Paleocene (around 70 MYA; Matsui et al. 2008; Zhang and Wake 2009). The sister taxa *A. japonicus* and *A. davidianus* likely diverged in the Pliocene (about 4.3 MYA) and are considered separate species despite a small degree of genetic differentiation (Matsui et al. 2008). The root of the *Cryptobranchus* mtDNA tree likely lies on the branch leading to the Current, Eleven Point, and New Rivers, and a common ancestor in the southern Ozarks and/or southern Appalachians is hypothesized to have given rise to all other populations, which is consistent with a Pleistocene refuge for this species as ice sheets covered the more northern regions until approximately 11,000 Before Present (BP) (Sabatino and Routman 2009).

In a recent study by Wiens et al. (2005), it was revealed that not simply the "presence" of "paedomorphic" characters, but rather the lack of clade synapomorphic characters were what misled phylogenetic analyses. This plasticity in the development of adult/terrestrial characters has allowed for convergence toward morphological/ecological specialization in the larval aquatic environment (which secondarily misleads reconstructions). Variation in the "larval" traits in these groups presents a special problem in that not all paedomorphic traits are shared across all clades/species (Wiens et al. 2005), with cryptobranchids presenting an adult skull more similar to those of other fully transformed salamanders (Duellman and Trueb 1994; Rose 1999; Rose 2003; Wiens et al. 2005).

Early morphology-based systematic studies placed Cryptobranchoidea as sister to all remaining salamanders, with the exception of the Sirenidae which are placed as basal on the phylogeny (Duellman and Trueb 1994). The classic study by Larson and Dimmick (1993), combining both molecular and morphological data, placed Sirenidae as sister to all extant salamanders and the early rRNA molecular dataset of Larson (1991) placed Sirenidae nested within the salamander tree. Current support for the basal placement of Cryptobranchoidea has come from molecular, morphological, and mixed datasets (Gao and Shubin 2001; Gao and Shubin 2003; San Mauro et al. 2005*; Wiens et al. 2005*; Zhang et al. 2005*; Frost et al. 2006; Marjanovic and Laurin 2007; Mueller 2006; Wang and Evans 2006; Roelants et al. 2007; Vieites et al. 2009; Pyron and Wiens 2011; * = subsets of analyses presented these relationships), while the basal placement for Sirenidae has come from morphology and some reconstructed phylogenies comprised of molecular and mixed datasets (Duellman and Trueb 1994; Larson and Dimmick 1993; San Mauro et al. 2005§; Wiens et al. 2005§; § = subsets of analyses presented these relationships).

Recent studies utilizing whole mitochondrial genome sequences (Zhang and Wake 2009) and mitochondrial genome and nuclear sequences (albeit, with limited taxon sampling; San Mauro 2010) placed Sirenidae as sister to all salamander families. San Mauro et al. (2005) placed (Sirenidae + Cryptobranchoidea) as sister to all other extant salamanders based on sequence from the 3' end of *Rag-1*. The characters analyzed (i.e., inclusion or exclusion of reproductive morphology and "paedomorphic" traits) and methodology used for phylogenetic reconstruction have played significant roles in the affecting the output of relationships; for this article we follow the Cryptobranchoidea placed basal on the phylogeny and Sirenidae sister to all other extant lineages (as in Vieities et al. 2009, Roelants et al. 2007, and Pyron and Wiens 2011). Nonetheless, we emphasize that deep salamander relationships are not clearly resolved at present.

Conservation genetics—Species and Evolutionary Significant Units

The basis of conservation genetics is identifying the genetic variation within a clade and within its comprising species, and consequently defining species and their genetic sub-populations in conservation categories as Evo-

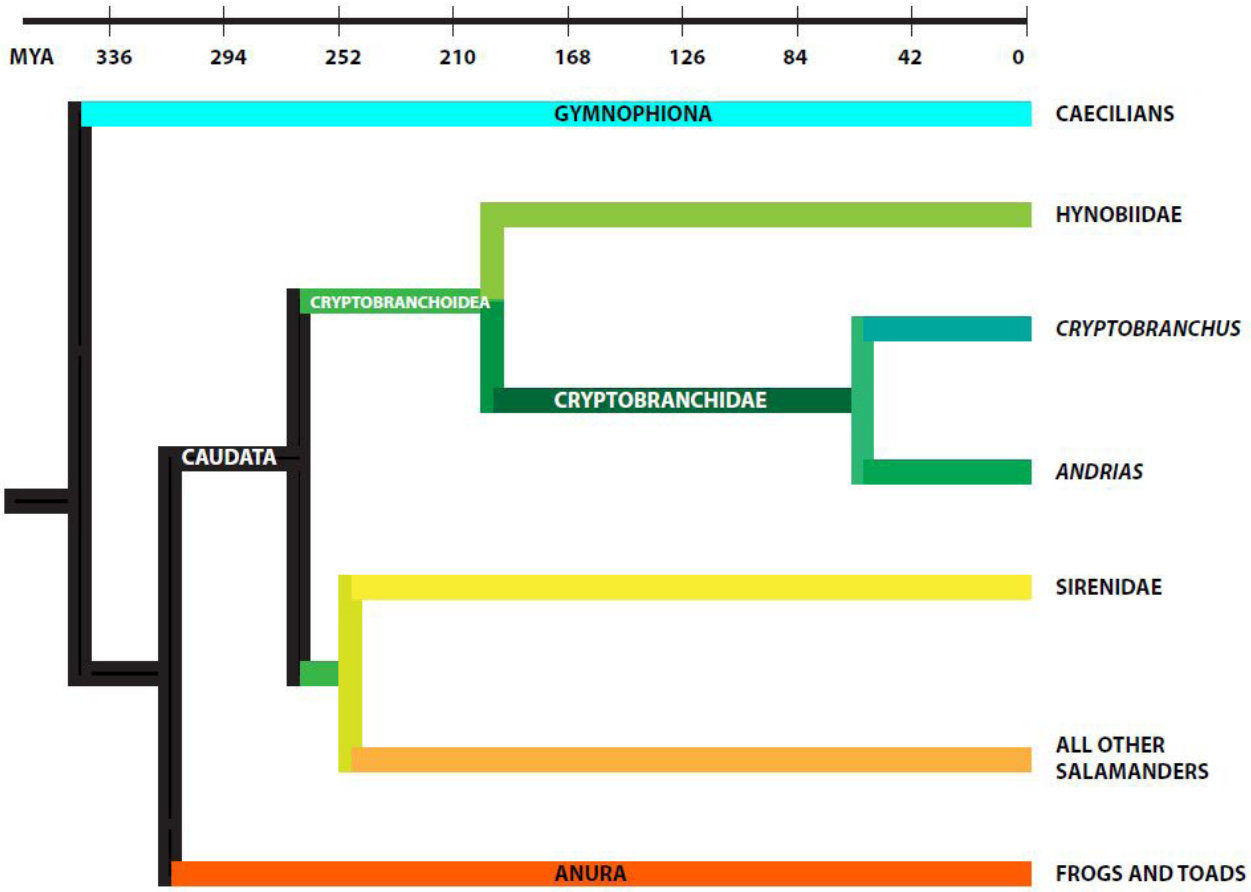

Figure 4. Phylogenetic tree showing ancestry of cryptobranchids and their hypothesized relationships to other amphibians. *Adapted from Roelants et al. 2007.*

lutionary Significant Units (ESU; sensu Wood and Gross 2008). This knowledge in combination with geography defines the range and distribution of species and their ESUs. This knowledge can then be used to perpetuate the genetic variation of the species through a range of practices based on the primary management unit, the ESU. An increasing focus on cryptobranchid conservation, and recent advances in genetic technologies, has resulted in a rapid increase in our knowledge of cryptobranchid conservation genetics.

The molecular techniques used to assess population structure, migration patterns, and their relationship to genetic variation, have rapidly progressed over the last 10 years. This progress has been largely driven by more rapid and cheaper sequencing and computer analysis, Information Technology systems, and a growing bank of molecular techniques and resources (GenBank 2012). Genetic variability in cryptobranchids has been defined with several types of molecular markers including allozymes, mitochondrial DNA (mtDNA) sequencing and

Figure 5 a, b. Taking tissue samples from tail clips (*Image: Amy McMillan*) or blood samples (*Image: Jeff Briggler*) enables conservation geneticists to assess an individual's relationship to other individual cryptobranchids and the relationship of its population to other populations of the same species.

Restriction Fragment Length Polymorphisms (RFLP), Amplified Fragment Length Polymorphisms (AFLP), and microsatellites. Older techniques used to estimate genetic structure and diversity, such as allozyme assays, required sampling whole organisms and may have negatively impacted population numbers. More recent Polymerase Chain Reaction (PCR) based techniques including AFLP, mitochondrial sequencing, and microsatellite markers take advantage of very small amounts of tissues that can be sampled without harm (Tanaka-Ueno et al. 2006).

For example, Foster (2006) collected small amounts of shed blood (amphibian erythrocytes are nucleated) when PIT tags were inserted subcutaneously, or sampled a small tail clip from *C. alleganiensis* that quickly regenerated. Blood samples also can easily be taken from the caudal veins of larger salamanders (see figure 5a). Tanaka-Ueno et al. (2006) found buccal swabbing was the most efficient non-invasive technique for sampling genetic material from caudata. Newer, non-invasive techniques, including environmental DNA (eDNA) sampling, have proven successful for detecting amphibian species in streams (Goldberg et al. 2011) and may prove useful for detection of cryptobranchids in natural habitats (Browne et al. 2011).

Mitochondrial markers have been used to resolve both inter- and intra-specific phylogenetic relationships as well as assess broad-scale population genetic structure. However, mtDNA is maternally inherited and so only tracks female lineages. Polymorphic microsatellite loci are typically found in non-coding or neutral regions within the genomic DNA, and their markers are currently the most commonly used genetic marker for studies of fine-scale population genetic structure in cryptobranchids. However, emerging methods for high-throughput genetic analysis promise to expand the scope of cryptobranchid conservation genetics to a genome-wide scale. Many areas of cryptobranchid research are likely to benefit greatly from ongoing efforts to obtain genome-wide nuclear sequence data, including transcriptome analysis (P. M. Hime, data not shown) and genomic analysis (R. L. Mueller, data not shown) in *Cryptobranchus*.

Polymorphic microsatellite markers can be robust and easily detected on either acrylamide gels or with fluorescence-based detection methods and are available for *Cryptobranchus a. alleganiensis* (Unger et al. 2010), *C. a. bishopi* (Johnson et al. 2009), *Andrias davidianus* (Meng et al. 2008; Yoshikawa et al. 2011), and *A. japonicus* (Yoshikawa et al. 2011). However, as the field of conservation genetics enters the genomic era, genome-wide molecular datasets will become increasingly available for cryptobranchids. These will enable deeper insights into their evolutionary history and cryptobranchid conservation genetics. Through using increasingly sophisticated genetic techniques phylogeny, paleogeography, species status, migration, effective population size, parentage, and population bottlenecking can eventually be known.

Andrias davidianus: Allozyme assays and mitochondrial DNA sequences revealed more variability in *A. davidianus* than in *A. japonicus* (Murphy et al. 2000). Tao et al. (2005) sequenced the mitochondrial control region of *A. davidianus* from the Yangtze, Yellow, and Pearl River regions and found low nucleotide and haplotype diversity within regions, especially the Yangtze River. Both of these studies showed very little differentiation in *A. davidianus* between regions. The population from the Huangshan area in China was genetically distinct from other areas, which suggests localized divergence, probably due to genetic drift and a lack of gene flow between this and other populations (Murphy et al. 2000). Despite the low genetic diversity, Murphy et al. (2000) found substantial substructure among *A. davidianus* populations but poor geographic correlation, even between the three major river systems in China. Nevertheless, Tao et al. (2005) discovered significant phylogeographic differences between the Pearl and Yangtze River regions, and between the Pearl and Yellow River regions. The genetic patterns discovered in these studies suggest that *A. davidianus* have a much higher gene flow between populations than either *A. japonicus* and *Cryptobranchus alleganiensis* (see below). Extensive human-mediated movement of *A. davidianus* may have begun over 3,700 years ago before the advent of historic Chinese Civilization by the Zhang Dynasty (3782-3058 BP; Ebrey 1996); the use of *A. davidianus* for medicine and food may have led to its human mediated transportation and thus may have facilitated this higher gene flow (Murphy et al. 2000).

Andrias japonicus: Early allozyme assays revealed little genetic diversity within *A. japonicus* (Matsui and Hayashi 1992). Mitochondrial DNA sequence variation is also relatively low but nevertheless indicates genetic subdivisions into central and western clades (Matsui et al. 2008). Matsui et al. (2008) noted that the low genetic differentiation in *A. japonicus* contrasted strongly with that of sympatric and also totally aquatic Hynobius species (Cryptobranchoidea). They suggested that the reduced genetic variability in *A. japonicus* may be attributed to polygyny by gigantic males with late sexual maturity and high longevity, a stable aquatic environment as habitat, as well as bottleneck effects during Quaternary glaciations (1.8 MYA to 20,000 BP). They suggested that the low genetic variation of *A. japonicus* may make the species prone to increased risk of extinction. Matsui and Tominaga (2007) found some nuclear genomic diversity in *A. japonicus* in a study of AFLPs but were not able to differentiate any geographic groups not identified with mtDNA methods.

Cryptobranchus alleganiensis: Early allozyme assays revealed very little genetic diversity across the range of *C. alleganiensis* (Merkle et al. 1977; Shaffer 1989). However, mtDNA RFLP and mtDNA sequencing studies revealed enough genetic diversity in *C. alleganiensis* to detect putative clades or management units (Rout-

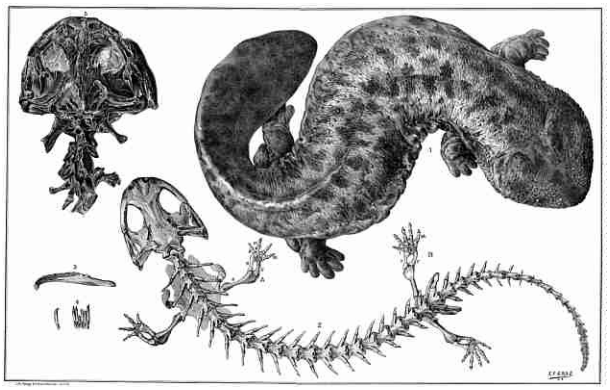

Figure 6. An early figure of Japanese giant salamander, *Andrias japonicus*, showing the dorso-ventrally flattened tail, the very broad head, and massive bulk of the *Andrias* species. The skeleton has remained almost unchanged for tens of millions of years. *Image from G. Mösch, Der Japanische Riesensalamander und der fossile Salamander von Oeningen, Neujahrsblatt der NGZH Nr. 89, 1887. Cryptobranchus japoniens Y. de Hoev. (Japanischer Riesensalamander.) Nach einer Photographie gezeichnet, in etwas mehr als 1/3 der natürlichen Grösse.*

man 1993; Routman et al. 1994; Sabatino and Routman 2009), a finding that was recently supported by nuclear microsatellite DNA markers (Tonione et al. 2011).

The monotypic genus *Cryptobranchus* has traditionally been divided into two distinct subspecies based on morphology and geography. The Ozark hellbender (*C. a. bishopi*) is only found in the Ozark Highlands of Missouri and Arkansas, whereas, the Eastern hellbender (*C. a. alleganiensis*) ranges throughout eastern North America; from eastern New York and Pennsylvania to the north and east, Mississippi, Alabama, and Georgia to the south, and Missouri to the west (Conant and Collins 1998). *Cryptobranchus a. bishopi* is characterized by large dark blotches on the dorsum and dark mottling along the chin, while *C. a. alleganiensis* has small spots on the dorsum and a uniform chin pattern (Petranka 1998). *Cryptobranchus a. bishopi* was described as a separate species by Grobman (1943), but current taxonomy recognizes the Ozark hellbenders as a subspecies.

Recent mitochondrial and microsatellite analyses have shown greater than previously recognized genetic variation in *Cryptobranchus*. These analyses suggest that this group is paraphyletic with respect to the currently recognized subspecies designations, and may potentially harbor unrecognized diversity. However, the species status of genetically distinct entities within this genus has yet to be examined in a comprehensive framework. Crowhurst et al. (2011) used nuclear microsatellite loci to show that *C. a. bishopi* is genetically distinct from *C. a. alleganiensis*, but that within the Ozark region there are two strongly supported groups that are as genetically distant from each other as each is from all *C. a. alleganiensis* samples combined. When the Ozark and Eastern hellbender samples were analyzed separately, the eastern samples resolved as two groups, albeit with weaker support than the Ozark sample distinction. This finding is not trivial for *Cryptobranchus* conservation. The Ozark subspecies was listed on the US Fish and Wildlife Endangered Species List in November, 2011 (US Government, 2011 No. FWS-R3-ES-2009-0009) and both subspecies have been included on Appendix III of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES).

Work by Sabatino and Routman (2009) using mitochondrial sequencing, and by Tonione et al. (2011) using microsatellite markers, recovered eight independent groups of *C. alleganiensis* which the authors advocated should be treated as separate ESUs. These are the Northern Ozarks, Ohio, and Susquehanna Rivers, Tennessee River, Copper Creek, North Fork of the White River, Spring River, New River, and Current/Eleven Point Rivers. These studies show that gene flow is severely restricted or non-existent among these eight major groups (as measured by the markers under investigation), and potentially among populations (rivers) within groups. Use of highly polymorphic microsatellite markers allows assignment of individual samples to specific management units. For example, Crowhurst et al. (2011) correctly assigned Ozark samples >91% of the time and a new Hellbender population in Georgia had an 84% probability of membership with an adjacent Tennessee River (Albanese et al. 2011).

Morphology and morphometrics

Andrias: The heads of *Andrias* are wide and flat reaching 1/5-1/4 of the snout-vent length. On their heads and necks, *A. davidianus* has paired small tubercles arranged in rows and *A. japonicus* large, single, and scattered tubercles. With both species tubercles are interspersed with abundant tiny sensory neuromasts that detect water movement and the presence of prey (Lannoo 1987). Their snouts are rounded with small nostrils near the snout tip, and their eyes are small and without eyelids. A labial fold is prominent at the posterior of the upper jaw. Their tongue with free lateral margins adheres to the mouth floor. Thick skin folds are present at the lateral side of the body and there are 12-15 costal grooves. All four limbs are short and stout with four fingers and five toes and lack skin folds or prominent interdigital webbing.

Tail length is between 59 and 80% of the snout-vent length. The dorsal fin of the tail is prominent and the ventral fin only conspicuous nears the vent (Fei et al. 2006). Coloration exhibits great variation. The skin of *A. davidianus* is dark brown, black or greenish and *A. japonicus* is reddish-brown with a paler venter; irregularly blotched and marbled with dusky spots (Chang 1936; Thorn 1969). Juveniles often have lighter coloration with small black flecks. Albinos (white or golden) have been recorded (Fei et al. 2006). There is no obvious sexual dimorphism in cryptobranchids, except during the breeding season when

mature males have an enlarged cloaca and females have a swollen belly when gravid (Niwelinski 2007). The larvae of *A. davidianus* have longer gills, their fingers and toes are more pointed, and their color darker than the larvae of *A. japonicus*. External gills disappear when total length reaches 170-220 mm (Fei et al. 2006).

Cryptobranchus: The head is strongly flattened, with small eyes and wrinkled fleshly folds of skin along each side of the body for respiration. Coloration exhibits great variation. The base coloration of *C. alleganiensis* ranges from grayish-black to tan and olive-green across the majority of the body (Nickerson and Mays 1973). The Ozark form *Cryptobranchus a. bishopi*, has many black blotches on the dorsum and the lower lips, while the dorsum of *C. a. alleganiensis* bears black spots rather than blotches, and the throat region may have pale spots (Petranka 2008). Albinos and morphs (orange to red patterns) have been occasionally observed (Dyrkacz 1981; Nickerson and May 1973; Fauth et al. 1996). *Cryptobranchus* retains a single pair of gill slits as adults unlike *Andrias*. Sexual dimorphism (enlarged cloaca in males and swollen belly in gravid female) is only obvious during the late summer to autumn breeding season. The larval stage of *C. alleganiensis* lasts 1-1.5 years during which they grow to 12.5 cm in length, gradually lose their external gills, and develop internal gills and a circular opening on each side to provide water for respiration, as well as development of fleshly fold along the sides of the body for respiration.

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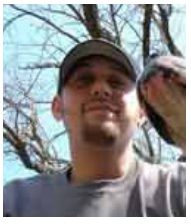

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In vitro culture of skin cells from biopsies from the Critically Endangered Chinese giant salamander, *Andrias davidianus* (Blanchard, 1871) (Amphibia, Caudata, Cryptobranchidae)

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***Abstract.*—We established a primary skin cell culture of the Critically Endangered Chinese Giant Salamander, *Andrias davidianus*, from small biopsies using minimal invasive methodologies. Biopsies were taken from three animals simultaneously with assessment of two biopsy sampling techniques using samples from the tail tip. Cell culture was performed in a wet chamber at room temperature. Several culture media and supplementations were tested as well as culture containers and surface coatings. The handling of *A. davidianus* in a landing net, without transfer out of the tank, allowed easier biopsy withdrawal. Best outgrowth of cells from explants was achieved in 60% DMEM/F12 medium with supplementation. Cells started to grow out as monolayer within the first 12 hours, and after three weeks formed pigmented multilayers, then died after 10 weeks. Primary cultures of *Andrias* skin cells, as well as other amphibian primary cell cultures, can be used in future studies to evaluate effects of disease, pollution, regeneration, wound healing, and could provide cells for use in reproduction technologies such as cryopreservation to preserve gene lines in this and other Critically Endangered species with minimal harm to the animals.**

Key words. Caudate cell culture, skin tissue explants, skin biopsy, biopsy withdrawal, amphibian skin cell culture, regeneration, wound healing

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Introduction

The Chinese giant salamander (*Andrias davidianus*) is the largest extant amphibian, with a total length of up to 180 cm. Together with the Japanese giant salamander (*A. japonicus*) from central and southern Japan, and the North American Hellbender (*Cryptobranchus alleganiensis*), these species form the sole members of the giant salamander family Cryptobranchidae, which is thought to be a basal family among caudate amphibians (Gao and Shubin 2003; review Browne et al. 2011). This family might be a survivor of a lineage that was already present in the Jurassic (Gao et al. 2003). The Chinese giant salamander is widespread in central, south-eastern and southern China, although its range is now very fragmented. The species inhabits streams and rivers in mountainous forested areas, at elevations from 100 to 1,500 m above sea level. Once common, the species has declined catastrophically over the last decades in their natural habitats while millions of these animals are bred in farms. Wild harvesting for human consumption is a major threat to *A. davidianus*, along with habitat destruction and degradation (IUCN 2012). Consequently, *A. davidianus* is now very rare in nature. *Andrias davidianus* is listed in Appendix I of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) and is also listed as Critically Endangered on the IUCN Red List of Threatened Species (IUCN 2012).

Research on diseases and other issues in salamanders, including *A. davidianus*, often involves sacrifice of the animals at the end of the experiments (e.g., Geng et al. 2011). An alternative to whole animal experiments that would minimize destruction of the animals is the use of *in vitro* cell cultures. Such assays have already been described for fishes. For example, Estepa et al. (1993) described a cell culture model to study the viral haemorraghic septicaemia virus in fin cells of rainbow trout *Oncorhynchus mykiss* (Estepa 1993). For this assay primary cultures from tissue explants of trout fins were established and infected with the virus *in vitro*.

The purpose of the present study was to determine whether it is possible to establish primary *in vitro* cultures of the skin cells of *A. davidianus* from small biopsies of tail tip tissue. Various cell culture media, surface

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Fig. 1. Biopsy method one performed in the zoo of Berlin. The animal was housed in an exhibition tank. It was captured and transferred in a tub, resulting in aggressive reactions of the animal, which made the biopsy procedure very difficult. A: animal housing. B: animal transferred into tub. C: handling of the animal to perform biopsies.

coatings and types of plastic containers were checked for cell outgrowth and long term survival. We find that this technique could serve as a feasible alternative to studies that require the destruction of individual animals.

Material and Methods

Three adult *A. davidianus* were used for this study. Sexing was done via ultrasound. One male was housed at the Berlin Zoo in an aqua-terrarium (L340 × W160 × H220 cm) with 50 cm water depth with shelter and decorative objects provided (Fig. 1A). Water temperature is 20 °C and water quality maintained by a sand-pressure filter, and partial daily, and complete weekly water changes. The remaining two adult *A. davidianus* were housed at the Cologne Zoo Aquarium. The couple is held in two concrete tanks (each L150 × W190 × H60 cm) with 50 cm water depth. The water (flowing water system) is connected to a cooling system and an external filter (OASE pond filter, Type Biotec Screenmatic) with a capacity of 10,000 L/h. Water parameters are as follows: temperature 20 °C, pH 7.3, conductivity 740 µS, carbonate hardness 7, and total hardness 16. Illumination is provided by T 26 fluorescent tubes (3 × 58 Watt). Tank roofing consists of stainless steel fence (1 cm mesh size), with one half being shaded each by styrofoam mats. Both tanks can be connected through a sliding gate (W60 × H60 cm) consisting of stainless steel wire (1 cm mesh size). The ground substrate consists of gravel and sand mixture with large roots. As hiding possibility, each tank contains a shelter (female tank: L80 × W50 × H50 cm; male tank: L125 × W50 × H50 cm) with entrance in front and exit at the rear side (each opening arched, W36 × H18 cm). Another adult male (not used for this study) is held in a tank in the public area of the Cologne Zoo Aquarium (L350 × W126 × H85 cm; temperature 14 °C, pH value 7.3, conductivity 668 µS, carbonate hardness 7, and total hardness 16; illumination: HQI spotlight, 400 Watt).

Biopsies

In order to keep the biopsy procedure as stress-free and efficient as possible two methods were tested. Method one (conducted at Berlin Zoo): 1) capture of the salamander, and 2) placing it in a tub with water and then taking biopsies from the tail tip (Fig. 1). Method two (conducted at Cologne Zoo): 1) Capture of the salamander in a landing net, and keeping it in its housing tank and taking biopsies (Fig. 2). Minimally-invasive biopsies were performed by using biopsy punches (Stiefel GmbH, Coral Gables, USA), with 4 and 6 mm biopsies taken from the tail tips of two males (Berlin

Fig. 2. Biopsy method two performed in the zoo of Cologne. Animals remained calm during the whole procedure and showed no reactions regarding handling of their tails. A and B: capture of the animal in a landing net. C and D: biopsy procedure at tail tip by use of biopsy punches. E: tissue inside a punch. F: transfer of tissue in tube with amphibian ringer solution for rinsing.

Table 1. Contents of Modified Amphibian Ringer Solution.

NaCl	100 mM
KCl	1.8 mM
$MgCl_2$	1 mM
$CaCl_2$	2 mM
HEPES	5 mM

and Cologne Zoo) and one female (Cologne Zoo). The procedure was performed without anesthesia as pain of biopsy is negligible, and consequently the risks of anesthesia too high. The procedure was classified as minimally invasive and performed in consent with the veterinary commissioner of the Cologne Zoo and the zoo's veterinarians. Giant salamanders are noted for their regenerative capacity, and consequently wound medication was not performed.

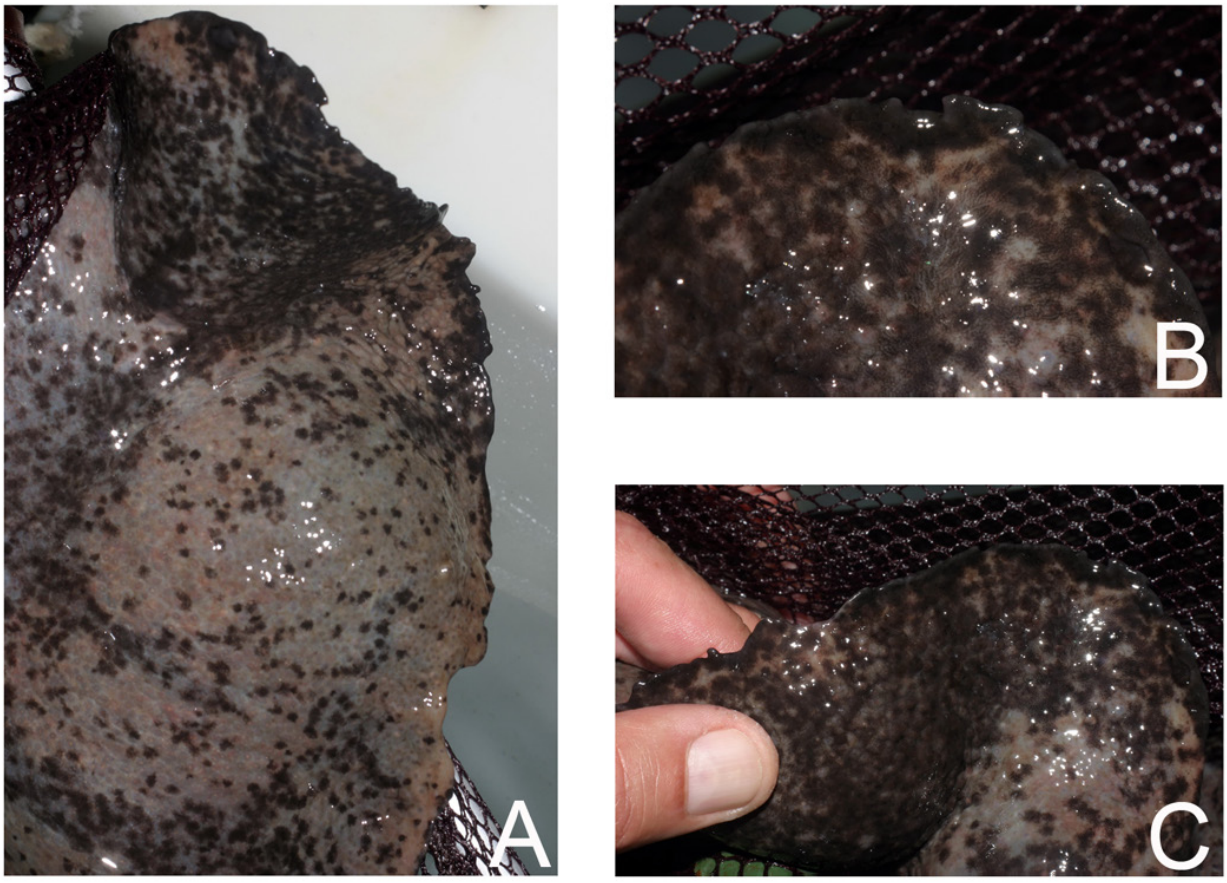

Fig. 3. *Andrias* tails nine months after biopsy procedures. Lost tissue was completely regenerated without scar formation or dyspigmentations. A: overview of tail. B and C: detail of tail tip.

Table 2. Cell culture media, supplements and coatings. Green highlight: optimal conditions for culture of *Andrias* skin cells.

Medium	**Supplements**							**Coating**			
	ITS	Sodium-P	NEA	A2P	P/S	Genta	HEPES	Collagen	PLL	FS	none
Williams Medium E	1%	1 mM	1%		50 U/ml	0.1 mg/ml	+				
	1%	1 mM	1%		50 U/ml	0.1 mg/ml			+		
	1%	1 mM	1%		50 U/ml	0.1 mg/ml				+	
	1%	1 mM	1%		50 U/ml	0.1 mg/ml					+
Leibovitz L-15	1%	1 mM	1%		50 U/ml	0.1 mg/ml	+				
	1%	1 mM	1%		50 U/ml	0.1 mg/ml			+		
	1%	1 mM	1%		50 U/ml	0.1 mg/ml				+	
	1%	1 mM	1%		50 U/ml	0.1 mg/ml					+
DMEM/F12	1%	1 mM	1%		50 U/ml	0.1 mg/ml		+			
	1%	1 mM	1%		50 U/ml	0.1 mg/ml			+		
	1%	1 mM	1%		50 U/ml	0.1 mg/ml				+	
	1%	1 mM	1%		50 U/ml	0.1 mg/ml					+
	1%	1 mM	1%	50 µg/ml	50 U/ml	0.05 mg/ml	5 mM				+

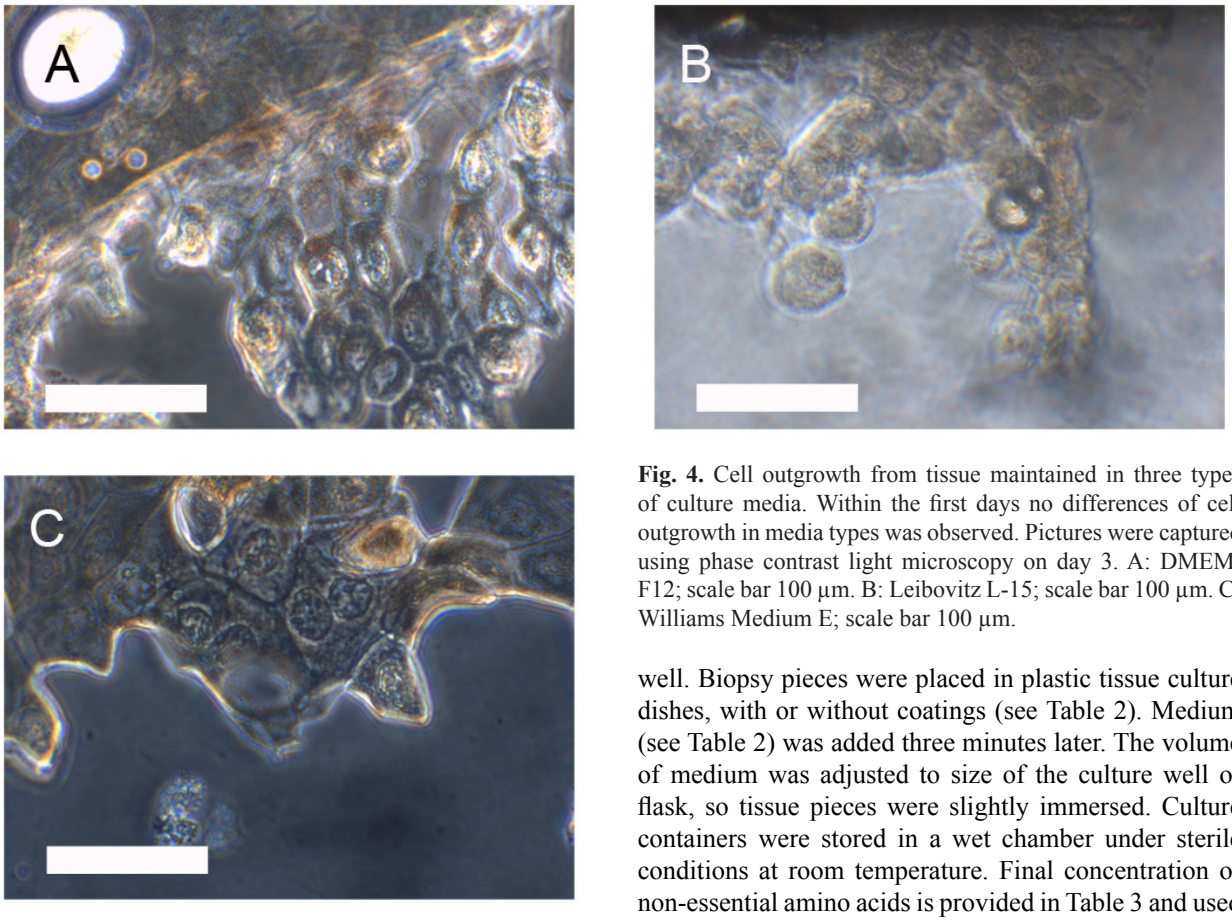

Fig. 4. Cell outgrowth from tissue maintained in three types of culture media. Within the first days no differences of cell outgrowth in media types was observed. Pictures were captured using phase contrast light microscopy on day 3. A: DMEM/F12; scale bar 100 µm. B: Leibovitz L-15; scale bar 100 µm. C: Williams Medium E; scale bar 100 µm.

Tissue Preparation

To reduce microbial contamination of cell cultures, biopsies were rinsed in 60% (v/v) PBS (phosphate buffered saline) or Amphibian Ringer Solution (see Table 1). These were salt solutions adapted to the osmolarity of amphibian cells, at pH 7. The solutions were supplemented with 50 U/ml penicillin/streptomycin (Biochrom) and 0.05 mg/ml gentamicin (Biochrom) to further support reduction of microbial contamination. Samples of one male *A. davidianus* (Berlin) were transported in cell culture media without supplementation for four hours. Samples of one male and one female animal (Cologne) were rinsed carefully, directly processed for cell culture without use of a cell culture workbench and after adherence transported to their storage place within three hours.

Biopsies were processed by cutting them into small (1-2 mm) pieces. As only small tissue samples were available, we decided to perform cell culture in small containers. The choice was between flasks that could be sealed thus making them suitable for transport of the culture from the zoo to the lab and multi well plates which are commonly used for cell culture assays. So 25 cm^2 tissue culture flasks were used especially for the starting cultures and 24 and 12 well plates were tested as well. Biopsy pieces were placed in plastic tissue culture dishes, with or without coatings (see Table 2). Medium (see Table 2) was added three minutes later. The volume of medium was adjusted to size of the culture well or flask, so tissue pieces were slightly immersed. Culture containers were stored in a wet chamber under sterile conditions at room temperature. Final concentration of non-essential amino acids is provided in Table 3 and used abbreviations and suppliers in Table 4.

Culture Containers

Following containers were examined for cell culture:

- 12 well plates, attachment surface of 3.6 cm^2/well (#92012, TPP, Trasadingen, Switzerland).
- 24 well plates, attachment surface 1.9 cm^2/well (# 92024, TPP, Trasadingen, Switzerland).
- Microflask, attachment surface 10 cm^2 (#91234, TPP, Trasadingen, Switzerland).
- Miniflask, attachment surface 25 cm^2 (# 90025 and 90026, TPP, Trasadingen, Switzerland).
- 24 well plates, attachment surface 1.9 cm^2/well (#CC7682, Cyto One, USA).
- Miniflask, attachment surface 25 cm^2 (#7.690, Greiner Bio One, Frickenhausen, Germany).

Depending on manufacturer´s production processes adhesion surfaces of the containers might be treated differently (e.g., plasma treatment of surfaces with varying protocols), resulting in varying adhesion conditions. As from mammalian primary cell culture is known that not every cell type adheres on every type of culture plastic, containers of various manufacturers were examined for cell culture of *Andrias* skin tissue explants.

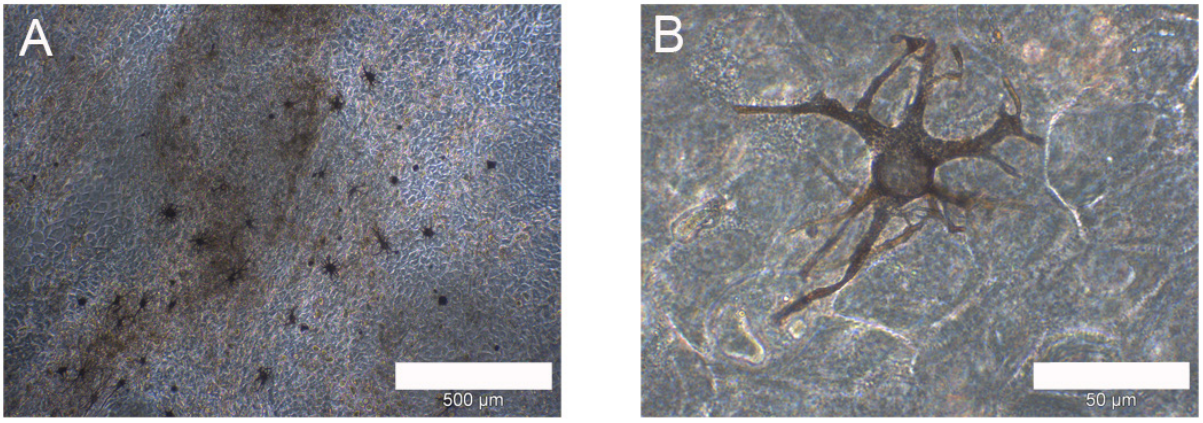

Fig. 5. Melanophores migrating from tissue. Skin epithelial cells and melanophores after two weeks of cultivation. The morphology of resident *Andrias melanophores* (A and B) appeared similar to observations of Billingham et al. in cytology of pigmented guinea pig skin. (C). Pictures were captured using phase contrast light microscopy. A: overview, migrating melanophores appeared rounded, whereas resident appear in the typical dendritic form; scale bar 500 µm. B: resident melanophore; scale bar 50 µm. C: resident melanophore (Billingham 1948).

Table 3. The final concentrations of non-essential amino acids and ITS in cell culture µg/ml.

L-alanine	8.9
L-asparagine*H_2O	15
L-aspartic acid	13.3
L-glutamic acid	14.7
Glycine	7.5
L-proline	11.5
L-serine	10.5
Insulin	10
Transferrin	5.5
Selenium A	0.0067

Media, Supplements and Coatings

All cell culture media were diluted to 60% (v/v) with sterile distilled water to achieve appropriate osmolarity. Media, supplementations and plastic coatings are listed in Table 1.

Cell culture material was coated by dropping solutions on the surfaces and drying under sterile conditions under a workbench, followed by three rinsing steps with sterile distilled water. Afterwards surfaces were dried again under sterile conditions. Coated surfaces were stored under sterile conditions at 4 °C for a maximum of one week. Media were changed twice a week. Cell outgrowth was digitally photographed with an inverse microscope and Cell D software (Olympus).

Results

Biopsy in A. davidianus

Method one resulted in aggressive reactions of the male that made taking of the biopsy difficult (Fig. 1). With method two both salamanders remained calm and did not react to the biopsy taking, which took less than five minutes (Fig. 2). Directly after biopsy the wounds bled sparsely or not at all, and inflammation and/or infection of the wounds did not occur. Healing took about two months; the lost tissue was completely regenerated without scar formation (Fig. 3).

Cell Culture

Cell culture was performed in a wet chamber at room temperature. Initially, technical difficulties had to be overcome resulting from low rates of adherence of the tissue fragments. In 12 and 24 well plates and microflasks, the tissue fragments adhered only in small proportions (5%), whereas more than 80% of the fragments adhered on the plastics of both types of miniflasks (Greiner and TPP). Cells started to grow out from adhered tissue under all culture conditions within 12 hours (Fig. 4). Beside skin epithelial cells also melanophores grew out. The melanophores appeared rounded during migration processes whereas resident cells showed typical dendritic morphology (Fig. 5).

Surface coatings did not result in better adherence or enhanced outgrowth. Interestingly, outgrowth from the female tissue appeared to be faster and spatially extended more than those from the males. Whether this observation is a general phenomenon or just occasional should be

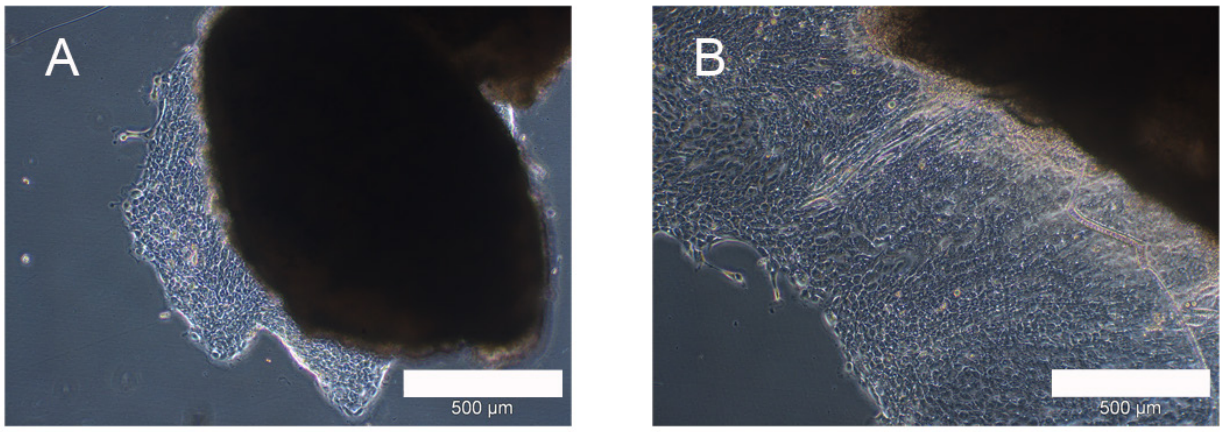

Fig. 6. Comparison of male (A) and female (B) tissue after three days of cell culture. Note that more outgrowing cells were observed in the female samples. A: male tissue at day three; scale bar 500 µm. B: female tissue at day three; scale bar 500 µm.

examined in further studies with higher numbers of tested individuals. In our study female cells grew out earlier and covered greater areas indicating faster rates of migration (Fig 6). Additionally, male cells became senescent earlier.

Influence of media conditions was tested in long-term culture. Cells in Leibovitz or WilliamsE cell culture media survived only for two weeks whereas cells with DMEM/F12 survived for 10 weeks. Cells grown in DMEM/F12 with full supplementation (see Table 2, green highlight) generally showed best results (Fig. 7). Cells grew out, formed complete monolayers and started to form tissue-like structures with pigmentation (Fig. 7 and 8). After six weeks multi nucleic cells occurred more frequently (Fig. 9), these cells stopped growing and finally died after 10 weeks. Dead cells broke away from the adhesion surface and floated in big sheets in the containers. Medium supplementation with HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) resulted in pH stabilization (visualized by phenol red indicator in cell culture media). Without this buffer medium's pH changed after less than one hour in the wet chamber as CO_2 fumigation was not available. With HEPES pH remained stable for up to two days. This short time of stability was caused by the low concentration of HEPES (5 mM) and small volumes of medium applied to the cells. Usually a concentration of 10 mM is used to stabilize media, but this concentration was found to be harmful to the cells of the giant salamander.

Problems with contamination by a fungus (white appearance, no determination of species performed) occurred in cell culture from one male animal (Cologne) and were treated with amphotericine B (Biochrome). This treatment stopped fungus growth, but cells started to age after two days of antifungal treatment. The cultures of the female (Cologne) and the other male (Berlin) tissues remained uncontaminated during the culture process. Repeated preparations from further biopsies of Cologne animals at later time points resulted again in fungal contaminations of male cultures.

Discussion

The large size and weight of adult *Andrias davidianus* make handling of the animals difficult and cause stress and possibly injury for both animals and researchers (e.g., bites, Beckstein 2009). To minimize such risks, we recommend using a landing net to restrain the animals in the housing tank for biopsy procedures as the animals stayed absolutely calm and apparently oblivious to the procedure (cf. Nickerson 2003; Mutschmann 2009).

We could find no literature concerning the cell culture of *A. davidianus* or any other cryptobranchid species in Western literature, or from correspondence through Chinese literature. Based on the cold freshwater physiological conditions experienced by *A. davidianus*, cell culture could be expected to be most successful with lower temperatures than with mammalian cells. Other conditions to consider with the establishment of *A. davidianus* cell cultures, in respect to those of mammals, are a lower osmolarity of body fluids in *A. davidianus* (Albert et al. 1987; Chernoff et al.1990), and particular cell culture coatings for optimal cell adherence and proliferation, as shown with *Xenopus laevis* and *Ambystoma mexicanum* primary cells (Nishikawa et al. 1990; Chernoff et al.1990). We assessed the use of different cell culture containers and various treated plastics (according to manufacturer's datasheets) combined with various media conditions and surface coatings.

We found that the size of cell culture containers was important for the successful outgrowth of cells, and tissue pieces were more likely to stick when small flasks were used instead of multiwell plates. This might be explained by the tendency of small pieces of tissue to float on the surface of solutions toward the containers wall thus preventing adhesion to the bottom of the container.

Cells from multicellular organisms communicate with each other by release of messenger substances into the extracellular fluids, e.g., the culture medium, or by direct cell-cell contacts. To accomplish sufficient concentrations

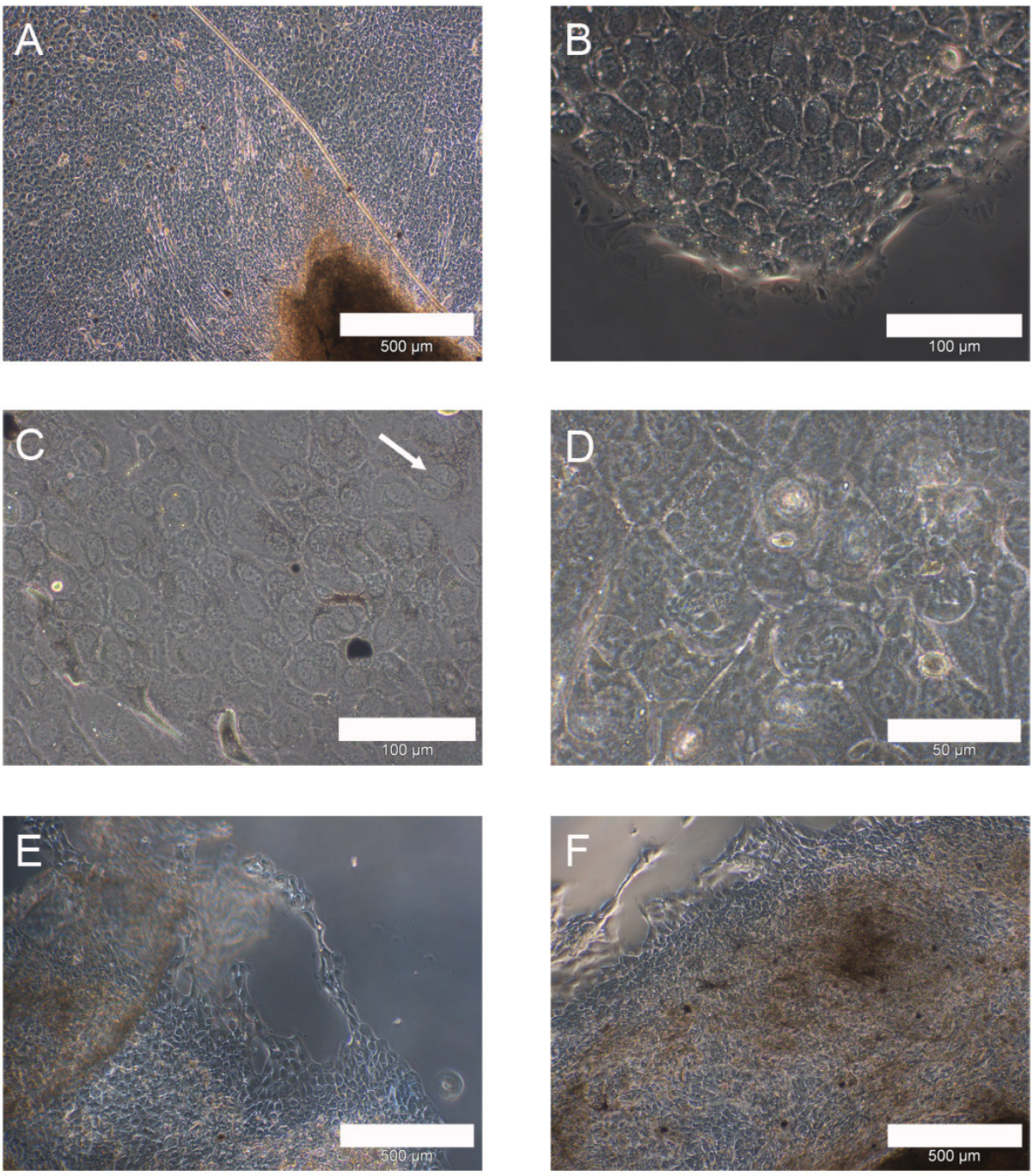

Fig. 7. Picture time line of cell outgrowth in DMEM/F12 with full supplementation. Images in overview and detail show representative examples of long term outgrowth of cells under full supplementation. Cells grew out in dense layers (A). At the migration front cells filopodia formation was observed (B). Outgrowing cells proliferated (C, indicated by arrow). No visual evidence for senescence was observed at day 18 to 25 (D, E, and F). After three weeks cells started to form pigmented tissue-like structures (E and F). Pictures were captured using phasecontrast lightmicroscopy. A: cells at day three; scale bar 500 µm. B: cells at day three; scale bar 100 µm. C: cells at day seven; scale bar 100 µm. D: cells at day 18; scale bar 50 µm. E: cells at day 21; scale bar 500 µm. F: cells at day 25; scale bar 500 µm.

of bioactive molecules by cellular release of substances like growth factors (e.g., vascular endothelial growth factor, keratinocyte growth factor, fibroblast growth factor), enzymes (e.g., lipoxygenases) and cytokines (e.g., interleukines) to their culture medium, low volume for small cell numbers is recommended. Too low concentrations of these substances lead to cell death *in vitro* as cells are missing paracrine stimulation. So the choice of cell culture container size means a balancing act between low surface curvature (implying use of greater culture containers) and low medium volume (implying use of smaller culture containers).

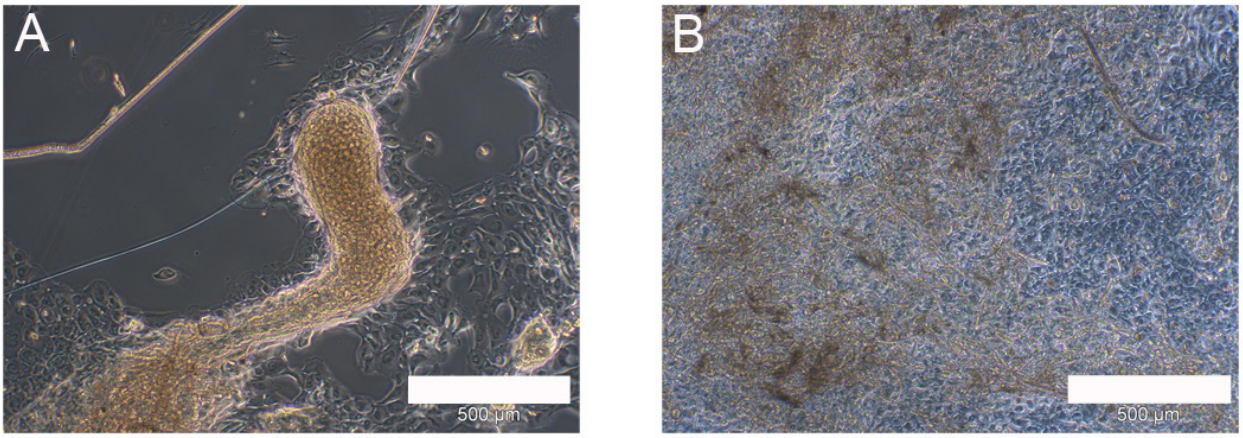

Fig. 8. Multilayer formations after six weeks of cultivation. Outgrowing cells tended to form pigmented multilayers with tissue-like appearance which became thicker with prolonged cultivation time. A: tissue-like structure after six weeks; scale bar 500 µm. B: tissue-like structure with pigmentation after six weeks of cultivation; scale bar 500 µm.

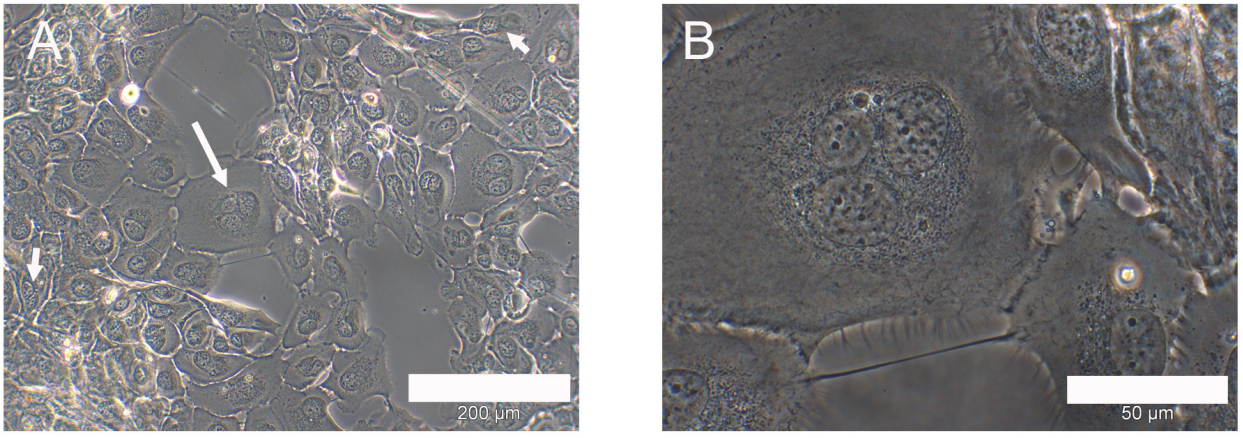

Fig. 9. Cell aging. After six weeks in DMEM/F12 multinuclear cells were observed more frequently. Pictures show representative examples and were captured using phasecontrast lightmicroscopy. A: overview (multinuclear cell indicated by arrows); scale bar 200 µm. B: detail of A; scale bar 50 µm.

Table 4. List of abbreviations and suppliers.

Williams Medium E		PAA, Cölbe, Germany
Leibovitz L-15		PAA, Cölbe, Germany
DMEM/F12		PAA, Cölbe, Germany
Ascorbate-2-phosphate	A2P	Sigma Aldrich, Taufkirchen, Germany
Insuline-Transferrine-Selenium A	ITS	Gibco
Non-essential aminoacids	NEA	Biochrom, Berlin, Germany
Sodium-Pyruvate	Sodium-P	Biochrom, Berlin, Germany
Penicilline/Streptomycine	P/S	PAA, Cölbe, Germany
Gentamicine	Genta	Biochrom, Berlin, Germany
Amphotericine B	Ampho	Biochrom, Berlin, Germany
(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid	HEPES	PAA, Cölbe, Germany
Collagen		Biochrom, Berlin, Germany
Poly-L-Lysine	PLL	Biochrom, Berlin, Germany
Fish Serum	FS	own production from trout blood

The influence of the adhesion surface on adhesion rates, cell migration, cell growth or the culture survival time seems to be negligible as no correlation to the cell culture material or surface coatings was observed. This is contrary to data from the literature describing culture of various amphibian cell types from *X. laevis* and *A. mexicanum* on developmental or regenerative aspects as well as toxicological studies (Albert at al. 1987; Nishikawa et al. 1990; Chernoff et al.1990; Goulet et al. 2003 et al.; Ferris 2010). In those studies cell culture vessel plastics were coated with fibronectin, collagen, matrigel and other matrices to encourage cell adhesion.

As nutrition media MEM, F12, MCDB151 or combination of these diluted to 70% with sterile distilled water were used (Nishikawa 1990). Culture media were supplemented with insulin, transferrin and EGF. Skin explant cultures obtained from *Ambystoma mexicanum* can be grown in 60% DMEM under supplementation with 10% fetal bovine serum and ITS (insulin transferrin, selenium A) (Ferris et al. 2010).

Culture survival appeared to be more dependent on the stabilization of culture medium pH than on surface coatings; mammalian primary cells usually need a stable pH around 7 to remain vital *in vitro*. Cells of *A. davidianus* were very sensitive to the supplementation with HEPES while the commonly used concentration of HEPES of 10 mM was toxic to the cells and led to cell death. A concentration of 5 mM resulted in stabilization of the pH as well as no detectable toxic influence on *A. davidianus* cells. High sensitivity to HEPES was also shown with a blastema model of A. mexicanum (Guelke et al. submitted). Previous publications on amphibian cells did not mention the use of HEPES in the culture media. Alternatively to HEPES, an incubator with CO_2 fumigation can be used to stabilize the pH (Chernoff et al.1990; Nishikawa et al. 1990; Ferris et al. 2010). Without pH stabilization cell outgrowth and survival was greatly reduced in our study as well as in other studies using CO_2 fumigation.

The benefit of the use of antibiotic supplements in amphibian cell culture may be negated by decreased survival. As caudates do not live in a sterile environment and need a certain skin flora, thus a problem rises with the transfer of tissue to cell culture; the culture medium offers good growth conditions for the target cells and simultaneously for microorganisms. Bacteria and fungi accrete faster than the cells and cause cell death by release of toxic substances. In our study, cells tolerated 50 U/ml of penicillin/streptomycin mix (p/s) which is sufficient to avoid infections of already established cultures. Therefore 0.05 mg/ml gentamicin was thus added. The common antibiotic supplementation of cell culture media contains 0.1 mg/ml of gentamicin, but this concentration resulted in early senescence and cell death of *A. davidianus* cells. There is no comparative research in Western scientific publications on the use of antibiotics in amphibian cell culture media.

The fungal contamination of the Cologne Zoos male's cell culture appeared to be from the skin microflora. Contaminations during tissue processing seem an unlikely cause as culture contaminations occurred under a wide range of preparation conditions including sanitized conditions. Further research is planned to identify the type of fungus and to assess its possible influence on outgrowth of cells from the tissue explants. *In vitro* treatments with amphotericin B for this fungus resulted in early senescence and cell death. Causes for this toxic effect remain unclear as amphotericin B (Fungizone) is commonly used in fish and amphibian cell cultures and known to be not toxic to cells so far. There is only one publication mentioning possible toxic effects of amphotericin B (Fungizone) on tadpoles of *Alytes cisternasii* (Martel et al. 2011).

Based on cell morphology we consider that outgrowing cells were skin epithelial cells and melanophores. Migrating melanophores appeared rounded while resident cells showed typical dendritic forms as these cells are from dendritic origin (Rawles 1948; Billingham 1948). In light microscopic imaging melanophores of *A. davidianus* appeared equal to those of guinea pigs shown in the study of Billingham (1948) which are compared in Fig. 5.

Interestingly, cell outgrowth from female tissue appeared to be faster than from male (Fig. 6). As we tested only samples of three animals so far, these observations need to be confirmed by repeating trials with other giant salamanders. From MRL mice it is known that females heal wounds better and faster than male animals due to sexually dimorphic genes (Blankenhorn et al 2003) and also with human cutaneous tissue (Gilliver et al. 2007), however we could find no published information on this phenomenon in fish or amphibians.

Cells did not only form a monolayer as known from primary cells in general, but tended to form pigmented multilayers in long term cultivation (Fig. 7F and 8) after three weeks. Usually mammalian primary cells stop proliferation when reaching confluence *in vitro* due to contact inhibition by cell-cell and cell-substrata interactions (Qi et al. 2008). In contrast most cancer cells or immortalized cell lines are refractory to contact inhibition and can continue to proliferate (Hanahan et al. 2000). Cell cultures from *Xenopus* skin explants only grew out as monolayer stopped expanding after six to eight days (Reeves et al. 1975). This raises the question whether the observed multilayer formation of *Andrias* skin explants could be related to the regenerative capacity of caudate amphibians.

Senescence is a well-known process in mammalian primary cells. Due to their limited proliferation capacity (Hayflick index) mammalian cells become senescent after certain time of *in vitro* cultivation in contrast to immortalized cell lines. Literature regarding life span of amphibian primary cells is limited and described results are ambiguous. While Nishikawa reports ageing

of *Xenopus* skin cells *in vitro* (Nishikawa 1990), Kondo et al. (1983) describes a growth crisis (senescence) in melanophores followed by a spontaneous transformation to an immortalized cell line derived from Rana catesbeiana (Kondo et al. 1983). In our study skin cells became senescent and did not undergo a spontaneous transformation and eventually died.

The creation of an immortal *Andrias* skin cell line could possibly be achieved by: 1) spontaneous transformation of cells as a small number of them undergoes a set of genetic alterations which lead to unlimited life span. This means, however, that very high numbers of primary cells may have to be cultivated over a long period of time until some of them start unlimited proliferation. 2) expression of telomerase reverse transcriptase (TERT) which e.g., is available as eukaryotic expression plasmid from ATCC (MBA-141). The use of method one is well documented in anurans (Kondo et al. 1983) as well as in fishes (review Lakra et al. 2011) while for method two only literature regarding fish cell lines is available (review Lakra et al. 2011).

Conclusion

This study examined the basic needs of primary cultures for *A. davidianus* skin cells raised from small skin biopsies. These cells seem to have no exceptional culture needs when cell culture is performed in a wet chamber except for specific medium osmolarity and pH stabilization with HEPES buffer.

Primary cultures of *Andrias* skin cells, as well as other amphibian primary cell cultures can be used in future studies to evaluate effects of; 1) diseases and effects of medication, 2) toxicity tests of pollutants and other substances as already described for fishes (Dayeh 2005) and anurans (Goulet 2003), 3) for the study of regeneration, and 4) the role of gender specific hormones on wound healing. The use of active or cryopreserved cell cultures, in conservation programs for threatened amphibians is being increasingly recognized. These cells can provide for the banking of cells and organelles, and their genetic material for use in reproduction technologies (Browne et al.). The next steps in the establishment of an *in vitro* cell culture model will be on the one side the development for cryopreservation cells do not have to be immortalized; they can be stored and cultivated we predict as mammalian primary cells.

A further contribution to cryptobranchid conservation of cell lines is their use for establishing of a karyogram based sex determination. Because of the large size of cryptobranchids sexing is often performed by ultrasonic examination, and due to the size of adult *Andrias* is an elaborate procedure. During ultrasonic examination which is usually done without anesthesia, also injury risks, both for animals and human beings, must be considered. Sexing with ultrasound is also most effective during the breeding period, when gonads are distinct and may effect reproduction. Based on the study of Zhu et al. (2002) *A. davidianus* may be distinguished by their sex chromosomes and this technique would enable a new less stressful sexing of these salamanders. Karyotyping also offers the opportunity to screen the animals for chromosomal aberrations to distinguish salamanders that may be unsuitable for use in conservation breeding programs. However, skin cell karyograms can only provide insights into chromosomal aberrations of somatic cells and not those induced by failures in the germ line. Examination of wound closure processes resulting from biopsy withdrawal *in vivo* and cell outgrowth *in vitro* could give information about the regenerative capacities of *A. davidianus*. Using cell culture models for *A. davidianus* research would reduce the number of experimental animals and provide new research horizons and benefit conservation breeding programs.

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